Université de Montréal
Faculté des études supérieures

Ce mémoire intitulé:

Activation of Expression of p15, p73 and E-Cadherin in Myeloid Leukemia Cells by Different Concentrations of 5-Aza-2'-Deoxycytidine

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a été évalué par un jury composé des personnes suivantes

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SUMMARY

Background: Inactivation of tumor suppressor genes plays an important role in leukemia. The inhibitor of DNA methylation, 5-aza-2'-deoxycytidine (5AZA), can re-activate these genes and is under clinical investigation for therapy of leukemia. However, the optimal dose/schedule is not known yet. The objective of this study was to determine the minimal concentrations of 5AZA that will re-activate target silent genes in human myeloid leukemic cell lines and correlate with drug cytotoxicity.

Materials and Methods: The effect of concentrations of 1 to 100 ng/ml of 5AZA on the re-activation of p15 and p73 in KG1a myeloid leukemic cells and E-cadherin in HL-60 myeloid leukemic cells was evaluated by RT-PCR. The amount of amplified DNA was quantified with an Agilent 2100 Bioanalyzer using 18S ribosomal RNA as reference standard. The mean values were compared using ANOVA. The effect of 5AZA on inhibition of growth, DNA synthesis and colony formation on these cell lines was also investigated.

Results: The extent of activation of the target genes was dependent on the concentration of 5AZA. For p15, activation was observed at 5 ng/ml or greater and for p73 and E-cadherin at 100 ng/ml of 5AZA. At these concentrations we also observed inhibition of growth, DNA synthesis, and clonogenicity. Gene activation and in vitro antileukemic activity increased with the concentration of 5AZA.

Conclusions: The in vitro antineoplastic and gene re-activation activity of 5AZA is dependent on the concentration of this analog and appeared at low concentrations in the range of 10 ng/ml.

Discussion: Translation from cellular models into clinical trials is complex. Demethylation of any specific gene has not yet been validated as a surrogate marker for cure. Based on our pre-clinical investigation, however, clinical trials should target a minimal plasma concentration of 5AZA in patients with leukemia in the range of 10 ng/ml.

Key Words: 5-aza-2'-deoxycytidine, myeloblastic leukemia, methylation, p15CDKN2B, E-cadherin, p73.

RESUMÉ

Introduction: L'inactivation de gènes suppresseurs de tumeurs joue un rôle clef dans la pathogénie de la leucémie. Le 5-aza-2'-deoxycytidine (5AZA), un inhibiteur de la méthylation de l'ADN peut réactiver ces gènes et est actuellement évalué dans le traitement des leucémies. Cependant le protocole d'administration optimal n'est pas encore connu. L'objectif de cette étude est de déterminer la concentration minimale de 5AZA capable de réactiver certains gènes inactivés par la méthylation dans des lignées cellulaires myéloides humaines et de corréler cette concentration avec la cytotoxicité du 5AZA.

Matériel et Méthodes: L'effet de différentes concentrations de 1 à 100 ng/ml de 5AZA sur la ré-activation des gènes p15 et p73 dans la lignée myéloblastique KG1a et de l'E-cadherine dans la lignée myéloblastique HL-60 a été évaluée par RT-PCR. La quantité de l'ADN amplifiée a été quantifiée par l'Agilent 2100 Bioanalyzer en utilisant l'ARN 18S ribosomal comme référence interne. La moyenne des valeurs a été comparée par ANOVA. L'effet du 5AZA sur l'inhibition de la croissance, la synthèse d'ADN et la formation de colonies a aussi été étudié.

Résultats: L'ampleur de l'activation de ces gènes a été proportionnelle aux concentrations de 5AZA. Pour le gène p15, l'activation a été observée à partir d'une concentration de 5 ng/ml. Pour les gènes p73 et l'E-cadherine il a fallu des concentrations de 5AZA supérieures à 100 ng/ml pour obtenir le même effet. Déjà aux basses concentrations de 5AZA, on a observé une inhibition de la croissance cellulaire, de la synthèse d'ADN et de la formation de colonies.

Conclusions: L'activité anti-néoplasique et la ré-activation génique du 5AZA est dépendante de la concentration de cet agent et s'observe déjà à d'aussi basses concentrations que de 10 ng/ml.

Discussion: La transposition en clinique de données obtenues in vitro est complexe. On n'a pas encore démontré que la démethylation d'un gène spécifique était corrélée avec la survie du patient. Cependant, en tenant compte de nos résultats, une concentration de 5AZA à partir de 10 ng/ml pourrait être recommandée pour les essais cliniques futurs de la leucémie.

Mots clés: 5-aza-2'-deoxycytidine, leucémie myéloblastique, méthylation, p15CDKN2B, E-cadherine, p73.

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ABBREVIATIONS

5AZA	5-Aza-2'-deoxycytidine
AML	Acute myeloblastic leukemia
ALL	Acute lymphoblastic leukemia
ARA-C	Cytosine arabinoside
BMT	Bone marrow transplantation
CML	Chronic myeloid leukemia
DNMT	DNA methyltransferase
CR	Complete remission
DLT	Dose limiting toxicity
HbF	Hemoglobin F
IC_{50}	Inhibitory concentration of 50%
HPLC	High performance liquid chromatography
$MeCP_1$ and $_2$	Methylated CpG binding proteins 1 and 2
MDS	Myelodysplastic syndrome
MTD	Maximal tolerated dose
PCR	Polymerase chain reaction

MSP	Methylation specific PCR
PR	Partial remission
RT-PCR	Reverse transcriptase polymerase chain reaction
TSG(s)	Tumor suppressor gene(s)

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DEDICATION

Dedicated to all those who wanted me to go one step further and had to work harder to allow me to do this research project.

PART I: BACKGROUND

CHAPTER I: ACUTE MYELOBLASTIC LEUKEMIA

Acute Myeloblastic Leukemia (AML) encompasses a heterogeneous group of diseases from a clinical, morphologic and a genetic point of view. However, except for the promyelocytic variant, the same type of treatment is employed for all. The standard treatment for AML is cytosine arabinoside (ARA-C) associated with topoisomerase inhibitors and anthracylines/mitoxantrone. For acute promyelocytic leukemia a similar regimen is used but all trans-retinoic acid is added.

In adult patients younger than 65 years the survival rate is only 40%[1]. In children this figure increases to more than 50%. The prognosis is better for the promyelocytic subgroup of leukemia.

Cure in leukemia is achieved with high morbidity due primarily to the severe toxicity produced by current chemotherapy. In some groups of patients the toxic death rate is very high and long-term effects are an unavoidable burden.

The prognosis in patients with recurrent disease is poor. There is an urgent need to test new drugs and regimens in order to improve the prognosis of this disease and reduce the expected side effects of the treatment.

CHAPTER II: EPIGENETICS

Epigenetics is defined as changes in gene expression that are caused by mechanisms that do not involve alterations in the DNA sequence[2,3].

The major epigenetic changes are methylation of cytosine nucleotides, in the promoter region of the gene and chromatin modifications such as acetylation of lysine residues in core histone. These two epigenetic phenomena are closely linked.

DNA methyltransferases are the main group of enzymes involved in methylation. They transfer a methyl group to cytosine using S-adenosyl-methionine as a methyl donor. DNA methyltransferase 1 (DNMT1) plays a fundamental role in methylation homeostasis (figure 2.1).

In mammalian cells 3% to 5% of the cytosine residues in genomic DNA are present as 5-methylcytosine[4]. Approximately 70-80% of the methylcytosine residues are found in CpG sequences[4].

In 1980, Razin et al[5] described the importance of DNA methylation as a key element in gene function and differentiation. It also has a major role in embryogenesis[6].

DNA methylation induces silencing of gene expression by inhibition of transcription. There are two main hypotheses which explain how DNA methylation inhibits transcription[7]:

1. It has been proposed that transcription factors bind less well to a promoter that contains 5-methylcytosine. However, this event alone does not explain the silencing of gene expression.

2. Other events are involved. Methylated CpG binding proteins $MeCP_1$ and $MeCP_2$, bind to the methylated promoter, which can also interfere with the

binding of transcription factors. A close link exists between DNA methylation and histone deacetylation (figure 2.1), the two major epigenetic mechanisms, which potentiate each other[8].

The relation between acetylation and deacetylation is fundamental in gene expression. Histones can be acetylated by histone acetyl-transferases and deacetylated by histone deacetylases. DNA methyl-CpG binding domain proteins and DNMT1 may recruit histone deacetylases to methylated promoters which in turn deacetylate histones to maintain chromatin in the repressed state.

The silencing of tumor suppressor genes (TSGs) can play an important role in cell cycle control resulting in unrestrained cell proliferation – a major trait in the definition of cancer.

Methylation of specific TSG and an increase at the level of mRNA of the DNA methyltransferases has been described in many tumors[9].

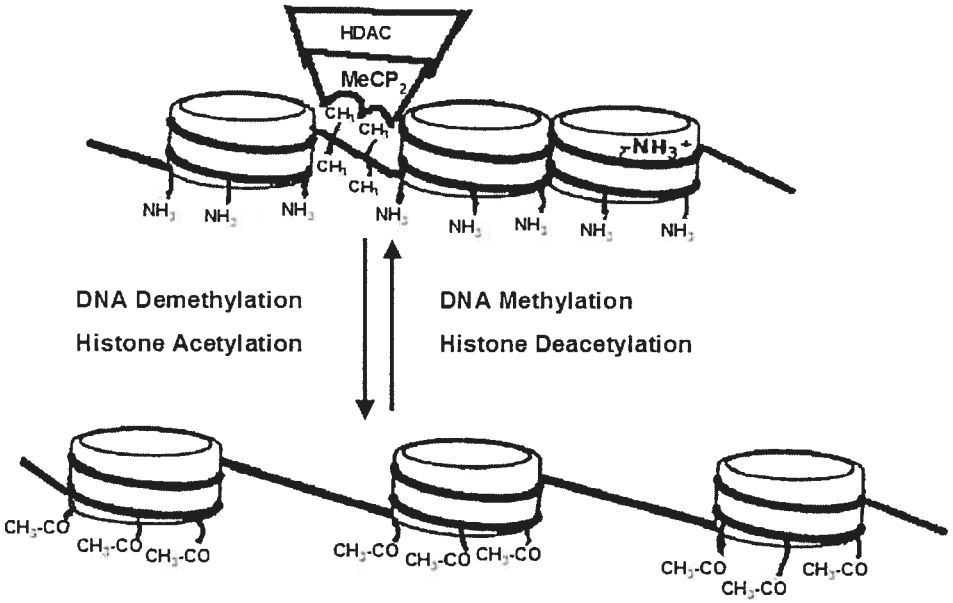


Figure 2.1: DNA methylation and histone deacetylation in the promoter region of a candidate gene. A nucleosome is shown. A methylated CpG site allows the binding of a 5-methylcytosine binding protein which recruits histone deacetylase and changes the configuration of the histones to a compact form preventing binding of the transcription machinery. Demethylation of the CpG site frees $MeCP_2$, transforming the histone structure into a loose form allowing transcription.

CHAPTER III: METHYLATED GENES

3.1 Methylated Genes in Cancer

TSGs can be silenced either by mutation, deletion or methylation. Genes involved in cancer invasion/metastasis suppression, DNA repair, angiogenesis, and genes coding for tumor antigens have also been described to be silenced by methylation in cancer[9].

3.2 Methylated Genes in Acute Myeloblatic Leukemia

Several genes are reported to be methylated in AML: p16INK4A, p15CDKN2B MDR1, MINT1, MINT 2, ER, WT1 and E-cadherin[10-12]. The methylation status of these genes can be used as markers to classify AML into different subgroups. It is not known which methylated genes play the most important role in cancer. p15 and p16 have analogous roles, p16 being more often deleted than methylated.

Among the methylated genes in myeloblastic leukemia p15, p73 and E-cadherin seem to be good candidate genes. They play roles in different cell pathways.

p15 gene (p15CDKN2B) is a gene located on chromosome 9p21. It encodes for a protein that inhibits cyclin dependent kinase 4 and 6 complexes. These D type cyclins, phosphorylate the retinoblastoma protein, which triggers the release of transcription factors that are necessary to enter S phase[13].

p15INK4B expression is usually inhibited by methylation. The tumor suppressor gene p15 is silenced by methylation in 61% to 100% of the myeloid leukemias[14].

Patients with ALL or AML with p15 methylation may have a worse prognosis[14,15]. Maloney described a possible role of the loss of function of p15

in the relapse of leukemia[16]. He reports that p15 became methylated in leukemic cells at recurrence, but not all authors confirmed this observation[17].

The p73 TSG is related to p53 and plays a role in DNA repair and apoptosis. It has been reported to be methylated in primarily lymphoid leukemia[18,19].

E-Cadherin is an invasion suppressor gene. Since leukemic bone marrow blasts migrate in the blood stream, E-cadherin might play an important role in the physiopathology of this disease. In a study by Melki et al[20], E-Cadherin was observed to be methylated in 78% of leukemic samples from patients.

3.3 Methylated Genes in Human Myeloid Leukemic Cell Lines

There are some human cell lines in which some genes are methylated and silenced.

In KG1a myeloid leukemic cell line, both p15 and p73 are completely methylated and not expressed[19; 21-23].

For HL-60 myeloid leukemic cell line, p73 is partially methylated whereas E-Cadherin is fully methylated[23,24].

CHAPTER IV: CANDIDATE GENES

p15, p73, and E-Cadherin are genes involved in leukemia and for which, we have cell lines where they are inhibited.

p15

p15 is in a region that is frequently mutated and deleted in a wide variety of tumors. Its mRNA sequence is described on the GenBank accession number NM_004936. p15 has a second isotype NM_078487 which contains an additional internal region which leads to a translation frame shift when compared to the first variant. It is located on 9p21. The gene is transcribed into a mRNA sequence of 2378 bp. p15 has a high amount of GC.

E-Cadherin (CDH1)

The mRNA sequence of E-cadherin is described by GenBank accession number NM_004360 and has 4828 base pairs. The E-cadherin gene is located on 16q22-1. It codes for an adhesion molecule of the cadherin superfamily, a calcium dependent cell-cell adhesion glycoprotein comprised of five extracellular cadherin repeats, a transmembrane region and a highly conserved cytoplasmic tail. Mutations in this gene are associated with gastric, breast, colorectal, thyroid, prostate and ovarian cancer[25,26].

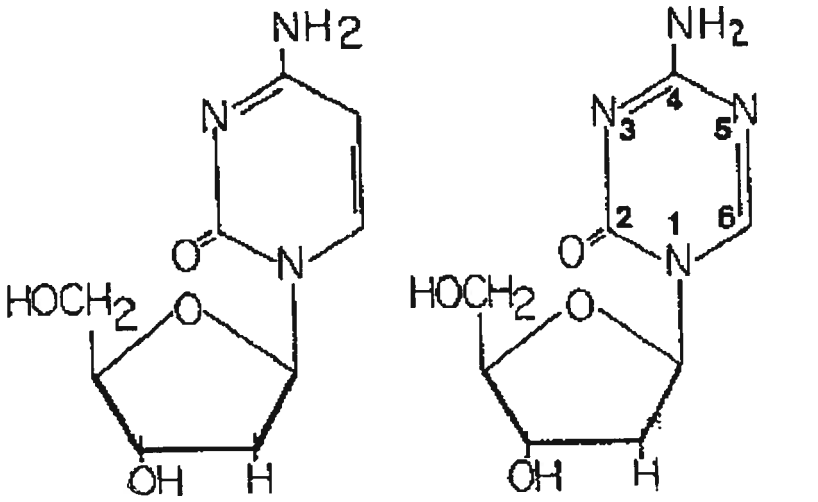
p73 Tumor Suppressor Gene

The mRNA sequence of p73 TSG is described by GenBank accession number NM_005427 and has 2234 bp. The p73 gene is located on 1p36.3. It is expressed both as multiple alternatively spliced C-terminal isoforms, and as N-terminally deleted, dominant-negative proteins that show reciprocal functional regulation.

CHAPTER V: 5-AZA-2'-DEOXYCYTINE

5-Aza-2'-deoxycytidine (5AZA) (Decitabine®, Dacogen®) is a chemical analogue of pyrimidine nucleotide 2'-deoxycytidine, the carbon of position 5 being replaced by a nitrogen group. Its chemical structure is shown on figure 5.1.

It was first synthesized in 1964 by Pliml and Sorm and demonstrated in 1969 to be a promising drug because of its antileukemic activity in animal models by Veshy and Sorn.



2'-deoxycytidine

5-aza-2'- deoxycytidine

Figure 5.1: The biochemical structure of 2'-deoxycytidine and its analogue 5-aza-2'- deoxycytidine

5.1 Mechanism of Action and Metabolism

5AZA is an analogue of deoxycytidine. Like the antileukemic drug, ARA-C, it is an antimetabolite with good cytotoxic activity. ARA-C is a potent inhibitor of DNA replication. The mechanism of 5AZA is different and related to its specific hypomethylation action which can reverse the silencing mechanism of methylation[27]. Figure 5.2 demonstrates its demethylating effect.

5AZA enters the cell, like the other nucleosides, both by an active transport system and by passive diffusion.

To be active, it has to be transformed into a triphosphorylated nucleotide by deoxycytidine kinase and other kinases[9]. It is incorporated into DNA by a reaction catalyzed by the DNA polymerase.

5AZA is metabolized by deoxycytidine-5-monophosphate deaminase and cytidine deaminase to pharmacologically inactive metabolites.

The antitumor activity of 5AZA is complex to evaluate because of the delayed antineoplastic action of the demethylating effect which induces terminal differentiation[9].

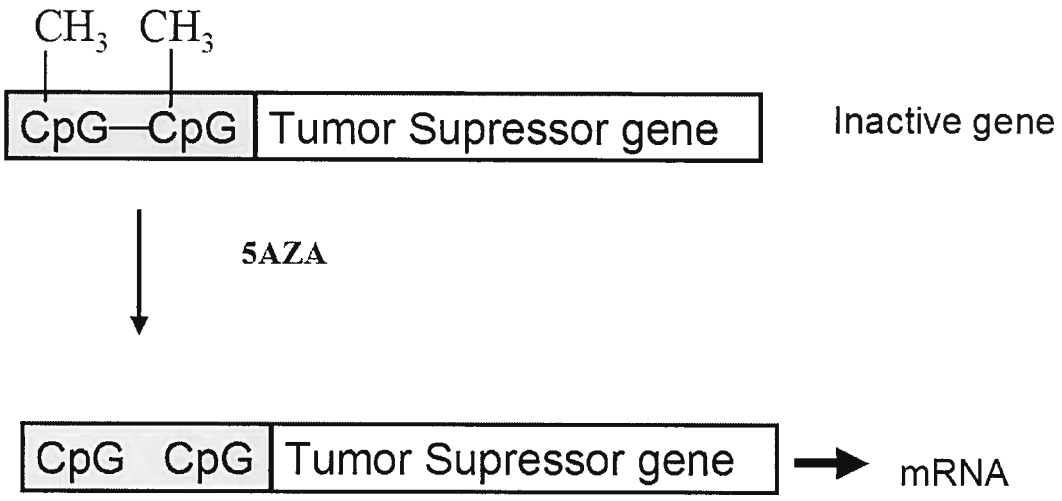


Fig 5.2: Demethylation of the CpG islands in the promoter region of the TSG by 5AZA. The demethylation of the CpG islands of the promoter region (shadowed) reactivates the target gene allowing the transcription into mRNA.

5.2 5AZA in Cellular Models

5AZA induces differentiation in mammalian cells[27], including myeloid leukemic cells[28,29].

In vitro it was more cytotoxic than ARA-C at the same concentrations[30].

For human leukemic cell lines, the minimal active concentrations of 5AZA were demonstrated by Momparler et al[31] to be in the range of 0.1μM (23 ng/ml) for 48h exposure.

In the human lung squamous carcinoma cell line SK-MES 1[32], 5AZA at a concentration of 100 ng/ml produced a loss of clonogenicity of 47.3% and 70% for an 8 and 24 h exposure respectively.

Cameron et al[33] demonstrated that concentrations of 1μM (228 ng/ml) showed marked demethylating activity after 1-5 days. Concentrations of 0.1μM (23 ng/ml) showed little effect on demethylation in the gastric cell carcinoma line RKO.

5.3 5AZA in Animal Models

5AZA showed potent activity in L1210 mice leukemia[34]. A 15 h i.v. infusion of 5AZA at a total dose ranging from 0.5 mg/kg to 22 mg/kg produced a high percentage of demethylation and an increase in the life span of the mice of greater than 150%. At higher doses, 5AZA showed curative potential of murine leukemia[35].

In mice[35], the antileukemic action of this drug is superior to ARA-C. There appears to be a good correlation between the extent of inhibition of DNA methylation and the antileukemic activity of 5AZA[34].

Pharmacokinetic studies in rabbits showed that after injection of 5AZA in a dose of 1.25 mg/kg over 15 minutes, the mean plasma levels were 3 μg/ml and after a dose of 2.5 mg/kg they were 7 μg/ml. In dogs they were 3 μg/ml at a dose of 2 mg/kg and 10 μg/ml at 4 mg/kg. 5AZA is quickly eliminated, with biphasic kinetics. The drug crossed the blood brain barrier, obtaining cytotoxic concentrations in the CNS of 50% of those observed in the plasma, but with a longer half life[36].

5.4 In Vitro Studies on Leukemic Cells from Patients and Anecdotical Cases

Momparler et al[37] investigated the effect of 5AZA on DNA methylation in the leukemic cells of 2 children, one with acute lymphoblastic leukemia (ALL) the other with chronic myeloid leukemia (CML) before and after treatment of 5AZA. They performed the same experiment on lymphoid CEM and HL-60 myeloid leukemic cell lines. The patient with ALL had recurrent disease and received a 24 h infusion of 5AZA (24 mg/kg) and the patient with chronic myeloid leukemia, received a 40 h IV infusion of 40 mg/kg of 5AZA. The cells were incubated in a medium containing 6-^{3}H-deoxycytidine. They analyzed the 5-methylcytosine ratio (5-methylcytosine/5 methylcytosine + cytosine) content by high performance liquid chromatography (HPLC) before and after treatment with 5AZA. At the end of therapy there was a reduction in methylation of around 80%. Exposure of HL-60 and CEM cells to 1μM of 5AZA for 24 h produced an 81 and a 68 % reduction in methylation, respectively.

Pinto et al[28] showed in vitro differentiation of leukemic blasts from patients, using concentrations of 5AZA of 1 μM added to the medium every 12 h for six times. He had previously reported that the optimal dose to induce differentiation in an erythroleukemia cell line was 1 μM (228 ng/ml) and that repeated pulses of 1 μM (228 ng/ml) up to 6 μM (1368 ng/ml) given every 6 hours produced maximal cell differentiation.

Gattei et al[38] studied the effect of 5AZA in acute myeloid leukemia cells from patients not eligible for aggressive chemotherapy and who were candidates to

receive 5AZA. In suspension the concentration of 5AZA required to reduce survival to 10% of the control cells was 30 μM (1.43-121.7) and to reduce to 50% was 9,1 μM (0.43 – 36.7)[1]. The same experiment was done with the cells on methylcellulose. With this solid medium lower concentrations were required to achieve reduction to 10% or 50% survival. They were 16 μM (0.9-81.1) and 4.8 μM (0.27-24.5) for the D_{10} and D_{50} respectively. There was no good correlation between the results obtained in the solid and liquid mediums.

4.5 Clinical Studies

PHASE I AND PHASE I/II STUDIES IN CANCER

Several Phase I studies were published with 5AZA. All the studies described, even those which are not designated as phase I/II provided data on the therapeutic response.

The first study by Rivard[39] published in 1981, was performed in children with leukemia (15 ALL, 7 AML) and 3 children with solid tumors (Wilms tumor, osteosarcoma, pulmonary blastoma). The starting dose was 0.75 mg/kg given as a 12 h infusion. The dose was escalated to 36-80 mg/kg as a 40-h infusion. At doses of less than 20 mg/kg, there was no observable toxicity. The dose limiting toxicity (DLT) was myelosuppression and the maximum tolerated dose (MTD) was 80 mg/kg. Other side effects observed were: alopecia, nausea, vomiting and diarrhea. In this study the response was also seen at doses less than 17 mg/kg but the antileukemic effect observed – reduction of leukemic blasts, bone pain, size of infiltrated testis was of short duration. The effect was longer at higher doses. Noteworthy was the clearing of cerebrospinal fluid and clinical improvement in 2 patients with meningeal leukemia.

In the subsequent studies, the MTD varied according to the study design and the DLT was consistently myelosuppression.

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[1]ie to reduce survival to 10% the concentration was 6,800 ng/ml (326 – 27,747 ng/ml) and to reduce to 50% the concentrations needed were 2,074 ng/ml (98-8,367 ng/ml)

In 1996 van Groeningen et al[40] published a phase I study with 21 adult patients with solid tumors. He administered 5AZA as a one-hour infusion separated by 7 h. Treatment was repeated every 3 to 6 weeks. He started with 25 mg/m^2 divided in two doses and the next steps were 50, 75, 90, 120, 180, 225 and 300 mg/m^2 divided in 3 doses. 5AZA was cleared very quickly from the plasma with t1/2α of 7 minutes and t1/2β of 35 minutes respectively. The DLT was myelosuppression, which increased markedly after 90 mg/m^2. There were also increases in creatinine. There was a partial response in one patient with a local recurrence of an undifferentiated carcinoma. All the other patients had short lasting stabilization or progression of the disease. The recommended dose was 75 mg/m^2 per infusion, with a total of 3 infusions given every 5 weeks.

In 1997 Momparler et al[32] published a phase I-II study of 5AZA in metastatic lung cancer. Fifteen patients entered the study and received a single 6-8 h iv infusion of 200-660 mg/m^2 repeated every 5-7 weeks. They showed some effect of the drug in this clinical setting. They also reported nausea and vomiting in approximately 80% of the cases, increased bronchial secretions in 20%, infection in 20%, mucositis in 13%, asthenia in 33%, loss of appetite in 20%, myalgia in 13% and in a small number of patients diarrhea, hiccups and dysuria. The causal relationship between 5AZA and the rare effects is difficult to establish. It is not always easy to distinguish the effects of the disease and those of the drug.

Another approach is to use low dose treatment to reduce hematopoietic toxicity and retain clinical efficacy. Preclinical studies show that demethylation occurs at low doses. Issa et al[41] published recently a phase I/II trial on 48 patients with refractory disease: 1 with ALL, 35 with AML, 7 with myelodysplasic syndrome (MDS) and 5 with CML. Two patients were treated twice; they had a stem cell transplantation and relapsed in-between episodes. Cohorts of 6 to 8 patients were treated with 5 mg/m^2 daily for 10 days (cumulative dose per cycle 50 mg/m^2) with a stepwise increase of 5 mg/m^2 to 20 mg/m^2 (cumulative dose per cycle 200 mg/m^2). After this phase two cohorts of 3 patients each were treated with 15 mg/m^2 for 15 and 20 days respectively (cumulative dose per cycle 225

mg/m^2 and 300 mg/m^2). Finally, a phase II trial was undertaken with 11 patients who received 15 mg/m^2 daily for 10 days to evaluate drug activity (cumulative dose per cycle 150 mg/m^2). The daily doses were infused over one hour. The cycles were repeated every 6 weeks. Overall objective responses were noted in 16 out of the 50 episodes (32%). In 9 (18%) a CR was observed. Among the 37 patients with AML, 5 (14%) achieved a CR and 3 (8%) had an objective response. In the Phase II group a response was observed in 6 of 11 patients (55%), 2 having a CR. The median time to response was 45 days (range 16 to 70 days). Eight of the 9 CRs occurred after 1 cycle. The drug was well tolerated. Even with these lower doses, myelosuppression was the major side effect. Grade II nausea, vomiting, diarrhea, skin rashes, liver dysfunction and creatinine elevation were also observed, as was grade III/IV hepatic toxicity. In such a study, DLT and MTD are difficult to evaluate. There was no correlation between p15 methylation at baseline or after therapy and response to 5AZA. However, for the methylation studies, peripheral blood was used without fractioning the blasts from the other cells, and the percentage of methylation was low. Furthermore the technique used to quantify methylation, COBRA, is not very sensitive.

Aparicio et al[42] recently reported a clinical trial of 5AZA administered by continuous infusion for 72 h at the dose levels of 20, 30, 40 mg/m^2/day in patients with metastatic solid tumors. The primary endpoint of the trial was to find a dose of 5AZA with acceptable side effects for clinical use. At 40 mg/m^2 Grade 4 neutropenia was seen in 1 of the 6 evaluable patients with 30 mg/m^2 and 2 of the 5 evaluable patients with 40 mg/m^2. This latter was considered unacceptable and Aparicio recommended 30 mg/m^2. The other toxic effects observed were fever, nausea, vomiting, constipation, weakness, anemia and thrombocytopenia.

The major toxic effect of the drug, myelosuppression, was used for Bone Marrow transplantation (BMT) conditioning[43] in patients with CML who relapsed after allogeneic related BMT. 5AZA was used as conditioning regimen with doses escalated in 3 steps from 100 to 150 mg/m^2 every 12 h for 5 days (cumulative dose 1,000 mg/m^2 to 1,500 mg/m^2). The regimen was well tolerated

with a response, either CR or PR, in 8 of the 14 patients with no patient having stage III/IV toxicity.

OTHER STUDIES IN CANCER

Several phase II studies with 5AZA either alone or in combination with other drugs have been published. The drug administration schedules and the overall responses seen in these studies are summarized in the table 1.1.

Testicular Cancer

Clavel[44] used 5AZA in 14 patients with non-seminomatous testicular cancer. A dose of 75 mg/m^2/dose was administered as a one-hour infusion 3 times a day. The schedule was repeated every 5 weeks. No activity was observed and the dose had to be reduced in 3 patients due to myelosuppression.

Chronic Myeloid Leukemia

In CML there are two studies by Kantarjian et al[45]: In 1997, they treated 37 patients (20 in the blastic and 17 in accelerated phase). They administered 100 mg/m^2 of 5AZA as a 6-hour perfusion every 12 h for 5 days (cumulative dose 1,000 mg/m^2). Because of prolonged myelosuppression, the dose schedule was subsequently reduced to 75 mg/m^2 in one-hour perfusion every 12 h for 5 days (cumulative dose 750 mg/m^2). The major toxicity was myelosuppression resulting in febrile episodes in 68% of the patients. The median time to recovery of granulocytes above 500/mm^3 was 48 days. Nausea and vomiting (12% of the patients), diarrhea (17%) and mucositis (7%) were also seen. Objective responses were noted in 25% of the patients in blastic phase and 53% of the patients in accelerated phase.

Recently Kantarjian et al[46] also confirmed the data in a bigger group. They administered cumulative doses of 1,000 mg/m^2 per cycle (subsequently decreased to 500 mg/m^2). 5AZA was given as a 100 mg/m^2 infusion twice a day for 5 days given over 6 h. Of 64 patients in CML blastic phase, 28% achieved

an objective response, 6 a complete hematological response. Among the 51 patients in the accelerated phase, 55% achieved objective responses. A median of 3 cycles was necessary to achieve the best response. The major side effect was myelosuppression.

Myelodysplastic Syndrome

Wijermans et al[47] using 5AZA at 45 mg/m^2/day given as a 4 h infusion every 8 h for 3 days every 6 weeks in 66 patients with myelodysplasia showed that 5AZA was effective in half of the patients, mainly those with the worst prognosis.

Daskalakis et al[48] demonstrated in vivo, the demethylation effect of 5AZA. He studied the p15 gene in high risk MDS patients treated with 5AZA, using bisulfite treated DNA PCR and sequencing. Among these 23 patients, 15 had hypermethylation of p15 gene and 9 of those had a decrease of methylation associated with clinical response.

Lubbert et al[49] and Wijermans et al[50] showed that low doses of 5AZA induced remission in elderly patients with MDS. Lubbert et al[49] studied the effect of 5AZA on the cytogenetics of patients with MDS in bone marrow samples from previous trials. These samples were taken prior to and after 5AZA treatment. He showed major cytogenetic responses in 19 out 61 patients with clonal chromosomal abnormalities and a reduced risk of death in patients achieving a major cytogenetic response. Their results suggested that 5AZA treatment in MDS might be linked to its ability to suppress the abnormal clone.

Acute Myeloblastic leukemia

Richel[51] treated 16 patients with relapsed leukemia, with 5AZA. Eleven were in their first relapse (10 AML, 1 ALL) and 5 unresponsive to high or intermediate dose ARA-C (4 AML, 1 ALL). The first group of 11 patients received 5AZA 125-250 mg/m^2 as a 6-hour infusion twice daily for 6 days, followed by Amsacrine 125-250 mg/m^2 as a one-hour infusion on days 6 and 7. The other group of 5 patients had 5AZA as monotherapy 250-500 mg/m^2 twice daily for 3-6 days.

The 11 patients who were in their first relapse of acute leukemia after a minimum of 6 months of complete remission had a good response to treatment. Eight (73%) achieved a complete remission and 2 a partial one. The second group did not respond to 5AZA alone. The major side effect described was myelotoxicity. Less frequent events included non-bacterial peritonitis and neurological complications like hemiparesis or somnolence. These appeared with higher dosages and were considered reversible.

Schwartsmann et al[52] used the combination of 5AZA and daunorubicin for the induction therapy of AML in adults. As first line therapy, they treated 8 patients with 4-hour infusion of 5AZA 90 mg/m^2, as first line treatment, daily days 1 to 5, associated with daunorubicin 50 mg/m^2 IV bolus, daily on days 1 to 3. One to 2 courses with a 5 to 6 week interval were administered. 6 patients achieved a complete remission after 1 (5 cases) or 2 cycles (1 case).

Petti et al[53] used 5AZA, 90-120 mg/mg^2 as a single agent given as a 4 h infusion in 12 patients with poor prognosis AML: 10 were fully evaluable, 3 achieved a CR and one a PR. Except for myelosuppression the toxicity was mild.

Gattei et al[38] treated 9 patients with AML, not eligible for standard treatment, with 5AZA 90-120 mg/m^2 as a 4 h infusion obtaining 2 CR and 2 PR.

Willemze et al[54] treated 63 adult patients with relapsed AML with 5AZA 125 mg/m^2 as a 6-hour infusion every 12 h for 6 days in combination with either amsacrine 120 mg/m^2 as one-hour infusion on days 6 and 7 (n=30) or idarubicin 12 mg/m^2 as a 15 min infusion on days 5 to 7 (n=33). Twenty-three patients (36.5%) obtained a complete remission after 1 or 2 cycles. Major grade III and IV gastrointestinal, cardiac, neurological and infectious toxicity was observed in a higher percentage of patients than what was seen with a classical induction in historical studies.

STUDIES IN SICKLE CELL DISEASE

5AZA has also been used in sickle cell disease to demethylate the γ-globulin gene inhibited by methylation.

A Phase I study[55] determined the effect of repeated 5AZA dosing on Hemoglobin F (HbF) levels and hematologic toxicity over a 9-month treatment period. Seven patients were entered. 5AZA was administered by intravenous infusion at a starting dose of 0.3 mg/kg per day, 5 days a week for 2 weeks, followed by a 4-week observation period. Average HbF and average maximal HbF levels attained during the last 20 weeks of treatment for the 6 SS patients increased to 13,93% +/- 2.75% and 18.35% +/- 4.46%, respectively, from a pretreatment mean of 3.12% +/- 2.75%. Mean and maximal hemoglobin levels increased from 7.23 +/- 2.35 g/dl to 8.81 +/- 0.42 g/dl and 9.73 +/- 0.53 g/dl, respectively.

Koshy et al[56] treated 8 adults with sickle cell diseases, 5 of whom were treated previously with hydroxyurea, with doses ranging from 0,15 mg/kg to 0,30 mg/kg given 5 days a week for 2 weeks. 5AZA was injected IV as a 4 to 7 minute perfusion. They observed, in this pilot study, an increase in HbF including an increase in patients previously treated with hydroxyurea.

Saunthararajah et al[57] used 5AZA 0.2 mg/kg 1 to 3 times per week in 2 cycles of 6 weeks duration with a 2 week interval between cycles. If there was a response, the dose was reduced in the next cycle. He used this scheme in 8 patients resistant or intolerant to standard treatment with hydroxyurea. The treatment increased the HbF and decreased the features of viscosity.

5.5 Dose Schemes Used in Phase II Clinical Studies in Cancer

The different schedules of 5AZA used in phase II clinical trials in patients with cancer are shown in table 5.1.

Pathology	Dose scheme	Perfusion time (hours)	Cumulative dose per cycle (mg/m^2)	Nber of cycles	Nber of patients	Objective Response (OR)	Reference
Relapsed AML	250-500 mg/m^2 bd (isolated) for 3 to 6 days 125-250 mg/m^2 (amsacrine) 6 days	6	1,500 – 6,000	1	16 (11 in the first relapse)	Associated with amsacrine 8 CR of the 11 in the first relapse 2 PR in the 5 others	Richel et al 1991[51].
Testicular cancer	75 mg/m^2 tid	1	225	q5 weeks	14	None	Clavel et al 1992[44]
AML	90-120 mg/m^2 tid for 3 days	4	810-1080	q4 to 6 weeks	12 (10 evaluable)	3 CR, 1 PR	Petti et al 1993[53]
AML	90-120 mg/m^2 tid for 3 days	4	810-1,080	Several 4-6 weeks	9	4 OR, 2 CR	Gattei et al 1993[38]
MDS	50 mg/m^2/day, 3 days 65 mg/m^2/day, 3 days	72	150 195	Maximum 8 q6 weeks	29	54% OR CR in 27 %	Wijermans et al 1997[50]
AML	90 mg/m^2 od, day 1 to 5	4	5-6 weeks	1 to 2	8 (6 evaluable)	Associated with daunorubicin CR in 6 patients	Schwartsmann et al 1997[52]
CML	75-100 mg/m^2 q12 h for 5 days	6	750-1,000	Several. Clinically indicated	37 20 in blastic phase 17 in accelerated phase	Blastic phase OR 25%, CR 10% Accelerated phase OR 53%	Kantarjian et al 1997[45]*
Relapsed AML or ALL	125 mg/m^2 q12 h for 6 days	6	1,500	»2	63	CR in 36,5% after one or 2 courses associated with either amsacrine or idarubicine.	Willemzee et al 1997[54]
MDS	45 mg/m^2/day in 3 divided doses x3	4	135	q6 weeks	66	OR 49% (OR 64% in patients with a high risk core)	Wijermans et al 2000[47]
CML	75-100 mg/m^2 q12 h for 5 days	6	750-1,000	several	130 64 in blastic phase 51 in accelerated phase	Blastic phase 28% OR, 6 CR Accelerated phase 55% OR 42% CR	Kantarjian et al 2003*[46]

* subsequently reduced to 750 mg/m^2 recommends 500mg/m^2 ie 50 mg/m^2 in q 12 hours for 5 days.

Table 5.1: Different dose schedules of 5AZA used in phase II clinical trials in cancer and the objective responses.

5.6 Clinical Pharmacokinetics and Pharmacodynamics

In a phase I study Rivard et al[39] used a bioassay based on growth inhibition of L1210 leukemic cells in cell culture. The steady state at the end of 24-40 h infusion was 500 ng/ml (2.1 µM) (range 300-900 ng/ml) (1.3-3.9 µM) when the infusion was normalized to 1 mg/kg/h.

Richel et al[51] with doses of 21 mg/m^2/h given as a 6 hour infusion twice daily had mean plasma concentrations of 0.6-1.2 µM (137-274 ng/ml). At dose rates of 42 mg/m^2/h and 84 mg/m^2/h the plasma levels were 2 µM (456 ng/ml) and 5 µM (1,140ng/ml) respectively. After discontinuation the plasma half-life was 8-14 minutes.

In a phase I study with solid tumors, van Groeningen et al[40] for the doses of 75 to 100 mg/m^2 obtained the peak plasma concentration of 207 ng/ml (0.93 µM) and 458 ng/ml (2.01 µM) respectively. In one case of 30 mg/m^2 and another of 60 mg/m^2 the peak plasma concentrations were determined to be 56 ng /ml (0.244 µM) and 93 ng/ml (0.409 µM). He used a bioassay based on growth inhibition in L1210 leukemic cells in mice.

Aparicio et al[42], studied a group of 16 adult patients with metastatic solid tumors. In two patients receiving 5AZA at 30 mg/m^2 over 72 h the plasma levels were 0,12 (27 ng/ml) and 0.16 μM (36 ng/ml). These are the lowest reported values associated with a biological effect: in vitro demethylation.

Momparler et al[32] in a phase I/II study in lung cancer examined the levels of 7 patients by HPLC. The steady state levels of the 100 mg/m^2 in 6 h were 390 ng/ml by HPLC and by bioassay, 310 ng/ml. For the 660 mg/m^2 in 8 h perfusion the mean concentration was 900 ng/ml (3.9 µM) by HPLC and 640 ng/ml (2.8 µM) by bioassay.

In CSF the concentrations of 5AZA were 20% of those in the plasma in patients with leukemia[58]

In a review, Momparler[59] mentioned that the effective cytotoxic concentration of 5AZA is probably around 1 μM (220 ng/ml). In vivo the optimal plasma concentrations for 5AZA are probably in the range of 0.1 - 1μM, the range of K_m of 5AZA[59]. Since the half-life of 5AZA is short 15-20 min[37] and the average cell cycle in AML is 60 h[31] with a range up to 90 h, Momparler suggested that the drug should be administered as a 90 h infusion.

Except for the results published by Aparicio[42], all 5AZA data was obtained using the endpoint cytotoxicity or differentiation, but not demethylation. Very few data exist about the necessary concentrations that demethylate the candidate genes.

PART II: OBJECTIVE OF THE STUDY

CHAPTER VI: OBJECTIVE OF THE STUDY

The main objective of this study was to investigate the minimum concentration of 5AZA that would produce activation of p15, p73 and E-Cadherin in human myeloid leukemic cell lines KG1a and HL-60.

A secondary objective was to investigate the effect of 5AZA on growth, DNA synthesis and clonogenicity with the same experimental conditions used to study gene expression and to correlate antineoplasic activity with gene expression.

Our hypothesis is that there is correlation between the concentrations required to re-activate gene expression and produce a significant antileukemic effect.

PART III. EXPERIMENTAL

CHAPTER VII: MATERIAL

5-aza-deoxycytidine (5AZA)

5AZA was obtained from Pharmachemie (Haarlem, The Netherlands) as a sterile powder. It was dissolved in phosphate buffered saline PH 7,4 and stored at -70º C. It was diluted in 0,45% sodium chloride on the day of the infusion and used immediately.

Cell Lines

Human myeloid leukemic cells HL-60 and KG1a were obtained from ATCC, Manassas, Virginia, USA.

CHAPTER VIII: CELL CULTURE AND IN VITRO TESTING OF 5AZA

8.1 Rationale

The antineoplasic effect of 5AZA can be tested in vitro in different ways. Clonogenic assays[60] have the greatest potential to predict the effect of the drug in vivo but are particularly time consuming. Other authors[61] confirmed this information. They showed in myeloblastic cells that the ability to clone in vitro is associated with a poor response to therapy in vivo. There are more rapid assays that can predict drug sensitivity like growth inhibition and inhibition of DNA synthesis[62].

8.2 Methods

To illustrate the effect of the drug: growth inhibition, DNA synthesis inhibition and clonogenic assays were performed under the same conditions. Cells were observed on microscopy regularly to look for any infection and their general aspect.

8.2.1 Cell Culture

Both cells lines were cultured in RPMI 1640 medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Wisent, St-Bruno, Quebec, Canada). The cells were incubated at 37°C in a 5% CO_2 atmosphere. All experiments were done when the cells were in good condition: weeks after unthawing, with an intact appearance on microscopy and having normal doubling times, ie 16-18 h for HL-60 and 27-30 h for KG1a.

8.2.2 Growth Inhibition

For growth inhibition, as for clonogenic assays and inhibition of DNA synthesis, the KG1a cells were treated with 5-AZA at time 0. No further drug was added and the medium wasn't changed. HL-60 cells were treated at time 0, 24 hours and 48 hours and the medium was changed. This was done according to previous experience in the laboratory. The cells were counted at 72 hours in both cases.

8.2.3 Clonogenic Assay

For HL-60 a clonogenic assay was performed by studying the ability of 5-AZA to inhibit colony formation. The cells were incubated with drug for 3 days then centrifuged and suspended in drug free medium. An aliquot of 100 cells was placed in 2 ml of 1.8 % soft agar medium containing 20 % serum. After 15 days incubation at 37ºC in 5 % CO_2 incubator the number of colonies (>500 cells) was counted.

The clonogenic assay was not performed for KG1a cells since this cell line does not form colonies in soft agar medium.

8.2.4 Inhibition of DNA Synthesis

The inhibition of DNA synthesis was measured by the incorporation of radioactive thymidine into DNA. Both cell lines were cultured in 5 ml flasks and treated at time 0 for KG1a and at time 0, 24 h and 72 h for HL-60. At 72 h the supernatant was discarded and the cells were diluted in 5 ml RPMI with 5% dialyzed serum. 1.25 µCi of [^{3}H] - Thymidine (ICN, Irvin, CA, USA) was added (5µl of 3H-TdR 1mCi/l diluted 1 in 4 in phosphate buffered saline) followed by CO_2 and the cells were incubated for 6 h. During that time the flasks were shaken manually every 30 minutes. The cells were then put on a GF/C filter, previously wet with 3 ml of 0,9% NaCl. The filter was washed with 3 ml of NaCl 0.9%. The cells were fixed with 3 ml cold 5% trichloroacetic acid. The filter was

washed with ethanol and dried during 10 min in an oven before being placed in ECOLite scintillation liquid (ICN). The radioactivity was measured using a Beckman LS 6000 IC scintillation counter.

CHAPTER IX: DESIGN OF PRIMERS

The primers are an essential part of the PCR. They anneal with each of the DNA strands and are fundamental in starting the reaction. The primers were designed to the candidate genes and house keeping genes.

9.1 House Keeping Genes

The house keeping genes are constitutional genes expressed in all the cells and used as internal controls for the PCR. We used β-2-microglobulin and 18S ribosomal genes.

β-2 Microglobulin mRNA is described on the GenBank accession number NM_004048 and has 925 bp. The β-2 microglobulin gene is located on chromosome 15. It codes for a 11 kDa protein associated with the outer membrane of many cells including lymphocytes. It is a small subunit of the major histocompatibility complex class I molecule.

18S ribosomal mRNA is described on the GenBank accession number X03205. and has 1869 bp. The 18S ribosomal gene is situated on chromosome 1. Its protein is fundamental for the protein synthesis machinery. It is highly expressed in all the cells and was used as our major internal standard. β-2 microglobulin was used as an extra control.

9.2 Ideal Primers

To have a maximal effect the primers should be 20-25 nucleotides long and have GC content of 40-60%. The higher the GC context the higher the annealing temperature. The T_m should be between 55ºC and 65ºC. The primers should not hybridize with each other to avoid primer dimmers. Preferentially the primers shouldn't have a T at the 3' terminus. The sequence of the primers

should be taken from different exons to avoid amplifications of genomic DNA during RT-PCR.

The primers for RT-PCR were designed using the nucleoside database. The RNA sequence was copied into the OLIGO program where it is analyzed and the best sequence for the primers chosen.

For each gene several primers were chosen and checked with positive controls, DNA existing in the lab where the candidate gene was known to be positive.

9.3 Selected Primers

Amongst the several primers analyzed we retained the following ones:

For β2-microglobulin sense 5' GAT GAG TAT GCC TGC CGT GTG A 3', antisense 5' CAA ACC TCC ATG ATG CTG CTT ACA 3' amplify a 94 bp product.

For 18S, sense 5' TCG ATG GTA GTC GCC GTG CCT A 3', antisense 5' CTG CTG CCT TCC TTG GAT GTG GTA 3' amplify a 110 bp product.

For p15, sense 5' CGC AGA CCC TGC CAC TCT CA 3', antisense 5' AGG CAT CGC GCA CGT CCA 3' amplify a 113 bp product.

For p73 two sets of primers were used. The first set were sense 5' GCA CCA CGT TTG AGC ACC TCT GGA 3', antisense 5' GAA TCC GTT CCG CCC ACC AC 3' amplify a 106 bp product and the second set, sense 5' CAG CCA CTG GTG GA 3', antisense 5' ACG GAG GGC AGC TTG TTC A 3' amplify a 134 bp product.

For E-Cadherin, sense 5' CAC AGG AGT CAT CAG TGT GGT CAC 3', antisense 5' CAC CTT GAA GGT CAG CAG CTT G 3', amplify a 92 bp product.

9.4 PCR Amplifications

For each gene the conditions for RT-PCR amplification and the reaction mixtures (25 µl) were found by trial and error in order to determine the best temperature conditions, the minimal number of cycles to give an amplification and the best mixture. All the mixtures contained 2.5µl of 1X PCR buffer, 1 µl of dNTP, 0.5 µl of each primer (2.5 µM) and 0.2 µl of Hot Start Taq polymerase (Qiagen) 5 units/µl. For the mixture the following solutions were tried when the primers didn't show a good amplification:

Q solution: a solution designed to modify the melting behavior of DNA

DMSO has been known to improve the amplification of some GC-rich DNA sequences, including some resistant to amplification by standard PCR techniques. It increases the amount of the product.

Mg_2SO_4 In addition to Mg^{++} ions bound by the template DNA, the nucleotides (dNTPs) and the primers, Taq DNA polymerase also requires free Mg^{++} ions. It influences primer annealing, the melting temperature of the PCR product and product specificity. An excessively high concentration leads to a reduction of specificity.

Solution Q was added in the reactions to p73 and p15.

The concentrations used were in accordance of Qiagen® recommendations.

When the attempts failed a new primer was designed.

The PCR amplifications were done under the following conditions: 95ºC for 15 min (activation of the Taq polymerase) followed by:

- For 18S: 94ºC for 45 s (denaturation), 60ºC for 30 s (annealing), 72ºC for 30 s (extending) for 5 cycles. Then the denaturation step was shortened by

15 s and the annealing temperature lowered by 2 degrees for 15 cycles. The PCR finishes with 10 min at 72ºC and 10 min at 10ºC.

- For p15: 94ºC for 45 s (denaturation), 62ºC for 30 s (annealing), 72ºC for 30 s (extending) for 5 cycles. Then the denaturation step was shortened by 15 s and the annealing temperature lowered by 1ºC for 35 cycles. The PCR finishes with 10 min at 72ºC.

- For p 73 (first set of primers): 94ºC for 45 s (denaturation), 62ºC for 30 s (annealing), 72ºC for 30 s (extending) for 5 cycles. Then the denaturation step was shortened by 15 s and the annealing temperature lowered by 2ºC for 37 cycles. The PCR finishes with 10 min at 72ºC and 10 min at 10ºC.

- For p 73 (second set of primers): 94ºC for 45 s (denaturation), 62ºC for 30 s (annealing), 72ºC for 30 s (extending) for 5 cycles. Then the denaturation step was shortened by 15 s and the annealing temperature lowered by 2ºC for 35 cycles. The PCR finishes with 10 min at 72ºC and 10 min at 10ºC.

- For E-Cadherin: 94ºC for 45 s (denaturation), 58ºC for 30 s (annealing), 72ºC for 30 s (extending) for 5 cycles. Then the denaturation step was shortened by 34 s and the annealing temperature lowered by 2ºC for 15 cycles. The PCR finishes with 10 min at 72ºC and 10 min at 10ºC.

CHAPTER X: RT-PCR

10.1 Rationale

Reverse transcriptase PCR (RT-PCR) is an *in vitro* method for enzymatically amplifying defined sequences of RNA in the form of cDNA.

When RNA is extracted the amount of RNA is measured by spectrophotometry. An equal amount of RNA is transformed by the reverse transcriptase in cDNA on which a classical PCR can be done.

10.2 Methods

After cell growth with different concentrations of 5AZA, total RNA was extracted, cDNA was made and the house keeping genes and the candidate genes were looked for.

10.2.1 RNA Extraction

The cells were harvested at 72 h and the RNA extracted using Absolutely RNA RT-PCR miniprep kit[63] (Stratagene, San Diego, CA, USA). After 72 hours of incubation with the drug, the cells were lysed using Lysis Buffer with β-mercapto-ethanol. Then the total RNA was extracted. All the material was RNAse free and the filters were protected with guanidine thiocyanate which destroys RNAse. The cells were successively filtered with 70% ethanol and different buffers prepared specially by Stratagene® for this effect. Then the homogenate was put on a fiber matrix and treated with DNAse. In this experiment we used DNAse Roche 10 U/ml, 2µl diluted in 5µl 10x DNAse buffer and 43 µl RNAse free water. It was incubated at 37ºC for 30 min. Again the homogenate was filtered through some buffers. Finally the homogenate which contained the RNA was placed in a fiber matrix and incubated with water which dilutes the RNA.

The amount of RNA in each sample is measured by a Beckman DU-600 photometer using the absorption for 3 light length λ260, λ280, λ230, the 2 latter to allow us to assess contamination.

10.2.2 cDNA

For cDNA synthesis[63], 500 ng of total RNA was reversed transcribed using OmniScript Rtkit (Qiagen, Missassaugua, Ontario). The reaction was performed at 37º C during 1 h followed by 5 min at 93°C to inactivate the enzyme. The mixture was prepared in 15 µl (7 µl of RNAse free water, 2 µl of 10 x Buffer RT, 2 µl of DNTPs 5 mM, 2 µl of hexanucleotide mix 10 x (Roche) 1 µl of RNAse inhibitor (10 U/ml) and 1 µl of Reverse Transcriptase 2U/µl. The reaction was performed with 20 µl – 15 µl of the mixture and 5 of a mixture of 5 µl RNA and water calculated to have approximately 500 ng of RNA per µl. A negative control was done with the same mixture but without Reverse Transcriptase.

10.2.3 PCR amplifications

The PCR amplifications were performed as described in chapter IX. The cDNA was checked for housekeeping genes β-2-microglobulin and the 18S ribosome, then the PCR was performed for the candidate genes.

CHAPTER XI: SEMIQUANTITATIVE MEASUREMENT OF GENES

An absolute quantification of the gene is not necessary when testing the expression of a gene after treatment with different concentration of a specified medication. In such studies the increase in gene expression is the studied endpoint.

We can relate the amount of DNA of the studied gene to a housekeeping gene; in this study, we used mainly the 18S ribosomal gene.

11.1. Ethidium Bromide Agarose Gel Electrophoresis

Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows detection of DNA fragments in gels.

After amplifying the cDNA, PCR for a housekeeping gene is performed. A known determined amount of the PCR reaction is put on a gel. After migration a photograph taken with a fluorescent light will reveal the amplitude of the migration band, which indirectly expresses the amount of DNA present. By trial and error, we find the amount of DNA product which will give the same band intensity on the gel after amplification. Since it is a housekeeping gene whose DNA is present in approximately equal amounts in all cells, we concluded that we therefore have the DNA from the same number of cells in each band. That amount will be used for the PCR for the studied gene.

The limit of detection for 1% agarose gel is 0.1 ng.

11.2 Capillary Electrophoresis and Detection of Fluorescence.

Capillary electrophoresis associated with fluorescent high performance liquid chromatography can be used to measure genes. This method was developed into an automated approach using the Agilent 2100 Bioanalyzer (Agilent

Technologies, Palo Alto, CA, USA)[64]. Chips determine the size and amount of DNA with high precision. Its limit of detection is 0.02 ng.

The amount of DNA amplified was analyzed by the Agilent 2100 Bionalyzer[65]. The amount of each sample was normalized to the DNA concentration obtained for 18S ribosomal gene. For each concentration of 5AZA the amount of cDNA which produced, after a PCR reaction, the same quantity (±10%) of 18s ribosomal gene was found. That amount of cDNA was used in a PCR reaction for the different TSG. To test the accuracy of the method used, a reference curve was generated repeating the measurement with the same PCR product 3 times and 3 times with different PCR products but with PCR done under exactly the same conditions.

11.3. Real-Time PCR.

Real time PCR[63,66] is a very precise method for detection and quantification of DNA. It has the advantage of detecting very small amounts of DNA.

There are two major Real-Time PCR methods: the standard curve method and the comparative cycle threshold method. In the standard curve method the amount of gene in the sample is calculated by comparison of a standard curve for the specific gene. The cycle threshold method detects the relative gene expression with a mathematical extrapolated formula.

In the standard curve method, which we used in the laboratory, the principle is a fluorescent dye like SYBR green which is added to the medium and the PCR product goes in a Real-Time machine which detects in "real time" the fluorescence of the dye associated with the DNA for each cycle, comparing it with the standard curve for the gene. The quantity of the gene present can be extrapolated.

Real-time PCR based on SYBR green fluorescence and the standard curve method was used for p15 with a Smart Cycler machine. The standard curve was done based on known amount of p15 measured in the Bioanalyzer.

CHAPTER XII: DIFFERENTIATION OF THE CELLS

12.1 Rationale

The demethylation effect of 5AZA has a slow mechanism of action. Differentiation could be assessed by microscopy or more accurately by quantification of neutrophil markers like CD11a,b,c and CD15[67]. This differentiation appears only weeks after treatment. Richel et al[51], in patients with ALL and AML, studied the cluster of differentiation before treatment and after 7 days of therapy. The loss of CD34 and the gain of CD33 in 3 patients was suggestive of differentiation.

12.2 Methods

We used microscopic morphology and immunocytochemistry to analyze the cells before treatment to assess the kind of cells we were treating and after 72 h. Samples of HL-60 and KG1a were taken of the first and the last days of treatment and colored with Wright stain and peroxydase.

To examine the cells on which we were working, we also imunophenotyped them at the beginning with the following monoclonal antibodies: CD33, CD13, CD15, CD11a,b,c. CD34, CD7, CD19, CD20 and CD2.

CHAPTER XIII: DATA ANALYSIS

All the experiments were repeated at least 3 times separated by at least one week.

For each of the genes, the mean for each dose is obtained and analyzed by one-way analysis of variance. The analysis is positive if at least 2 means are different. To identify the mean which is different we used the multiple comparison method with the SIDAK t test correction which has tighter bounds than the Bonferroni test. The analysis were done using the SPSS 12 software.

Given the fact that the relative values obtained with Bioanalyser are standardized for the 18s ribosomal gene our main analysis was done on the basis of these values. We also did the same analysis on the ratio p15/18s and examining the increase of p15 and of this rate above the baseline.

PART IV: RESULTS

CHAPTER XIV: MANUSCRIPT: ACTIVATION OF EXPRESSION OF P15, P73 AND E-CADHERIN IN LEUKEMIC CELLS BY DIFFERENT CONCENTRATIONS OF 5AZA

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Activation of Expression of p15, p73 and E-Cadherin in Leukemic Cells by Different Concentrations of 5-Aza-2'-Deoxycytidine (Decitabine)

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Abstract. *Background: Inactivation of genes that suppress neoplasia by aberrant DNA methylation is a key event that occurs during the development of leukemia. The inhibitor of DNA methylation, 5-aza-2'-deoxycytidine (5AZA), which can re-activate these genes, is under clinical investigation for therapy of leukemia. The objective of this study was to determine the concentrations of 5AZA that will re-activate target silent genes in human leukemic cell lines. Materials and Methods: RT-PCR was used to evaluate the effect of concentrations of 1 to 100 ng/ml of 5AZA on the re-activation of p15 and p73 in KG1a myeloid leukemic cells and E-cadherin in HL-60 myeloid leukemic cells. The effect of 5AZA on inhibition of growth, DNA synthesis and colony formation in these cell lines was also investigated. Results: The extent of activation of the target genes was dependent on the concentration of 5AZA. For p15, pronounced activation was observed at 10 ng/ml or greater. For p73 and E-cadherin significant activation was observed at 100 ng/ml of 5AZA. Maximal inhibition of growth, DNA synthesis and colony formation occurred at 100 ng/ml. Conclusion: The in vitro antineoplastic and gene re-activation activity of 5AZA is dependent on the concentration of this analog. These data may be helpful in the design of the optimal dose-schedule of 5AZA for the clinical therapy of leukemia.*

The silencing of the expression of genes that suppress neoplasia by an epigenetic event, such as aberrant DNA methylation, can play an important role during the development of malignant disease (1). The cyclin kinase inhibitor gene, p15CKN2B, the p53 gene homologue, p73, and the cell adhesion gene, E-cadherin, have been reported to be silenced by DNA methylation in leukemia (2-4). 5-Aza-2'-deoxycytidine (5AZA), a potent inhibitor of DNA methylation, can re-activate the expression of many genes silenced by promoter hypermethylation (5). In phase I-II studies 5AZA was shown to be an active agent for the therapy of hematological malignancies (6-9). Several clinical trials on 5AZA are currently underway in patients with leukemia. Preclinical studies of this analog can provide data that can be helpful in the design of optimal dose-schedules of 5AZA for clinical trials (10).

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Key Words: 5-aza-2'-deoxycytidine, leukemia, methylation, p15CDKN2B, E-cadherin, p73.

Materials and Methods

Materials. 5AZA (Dacogen, Decitabine) was obtained from Pharmachemie (Haarlem, The Netherlands). Stocks were made in phosphate-buffered saline pH 7.4 and stored at -70°C. HL-60 and KG1a human myeloid leukemic cells were obtained from ATCC, Manassas, Virginia, USA. Both cell lines were cultured in RPMI 1640 medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Wisent, St-Bruno, Quebec, Canada). The cells were incubated at 37°C in a 5% CO_2 atmosphere.

Drug treatment and in vitro assays. For KG1a, different concentrations of 5AZA were added to the medium once at time 0. For HL-60 leukemic cells 5AZA was added to the medium at time 0, 24 and 48h. For the growth inhibition assay, cells were counted at 72h using a model ZM Coulter counter. For clonogenic assay, an aliquot of 100 cells of HL-60 was placed in 2 ml of 3% agar medium containing 20% serum. After 15 days incubation at 37°C in 5% CO_2 the number of colonies (>500 cells) was counted. The rate of DNA synthesis was measured by the incorporation of radioactive thymidine into DNA. At the end of drug treatment, the cells were centrifuged and suspended in 2 ml of medium containing 5% dialyzed serum and 0.5 μCi [3H] thymidine (6.7 Ci/mmol, ICN, Irvin, CA, USA) and incubated at 37°C for an additional 6h. The cells were placed on GF/C glass fiber filters (2.4 cm diameter), washed with cold 0.9% NaCl, 5% cold

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Table I. *Growth inhibition and loss of clonogenicity by different concentrations of 5AZA on HL-60 and KG1a myeloid leukemic cells.*

5AZA concentration (ng/ml)	HL-60 growth inhibition (%)	KG1a growth inhibition (%)	HL-60 loss of clonogenicity (%)
1	3.5 ± 5.1*	27.8 ± 3.2*	31.3 ± 1.1*
5	nd	56.5 ± 3.3	nd
10	22.3 ± 8.9	59.6 ± 3.7	60.8 ± 11.2
50	36 ± 8.8	nd	100 ± 0
100	41.9 ± 11.5	67.5 ± 3.0	100 ± 0

Drug exposure 72 h. *mean ± SD; n = 3-7. nd, not determined.

Table II. *Inhibition of DNA synthesis by different concentrations of 5AZA on HL-60 and KG1a myeloid leukemic cells.*

5AZA concentration (ng/ml)	HL-60 inhibition of DNA synthesis (%)	KG1a inhibition of DNA synthesis (%)
1	5.5 ± 5.1*	25.6 ± 5.3
5	nd	43.8 ± 13.4
10	20.0 ± 13.5	51.7 ± 21.4
50	49.5 ± 7.8	nd
100	52.1 ± 23.2	74.2 ± 10.8

Drug exposure 72 h. *mean ± SD; n = 3. nd. not determined.

trichloroacetic acid and ethanol. The filters containing the DNA were then dried, placed in scintillation liquid and the radioactivity measured using Beckman LS 6000IC scintillation counter.

Isolation of RNA and RT-PCR analysis. After drug treatment the cells were harvested and total RNA and cDNA prepared as previously described (11). PCR was performed using HotStar Taq DNA polymerase (Qiagen, Mississauga, Ontario, Canada) and specific primers. The primers for 18S ribosomal RNA (GenBank accession no. X03205) were sense 5'- TCG ATG GTA GTC GCC GTG CCT A- 3', antisense 5'- CTG CTG CCT TCC TTG GAT GTG GTA- 3' which amplify a 110 bp product. For p15 (GenBank accession no. NM _4936) the primers were sense 5'- CGC AGA CCC TGC CAC TCT CA- 3', antisense 5'- AGG CAT CGC GCA CGT CCA- 3' and amplify a 113 bp product. For p73 (GenBank accession no. NM _005427) the primers were sense 5'- GCA CCA CGT TTG AGC ACC TCT GGA- 3', antisense 5'- GAA TCC GTT CCG CCC ACC AC- 3' and amplify a 106 bp product. For E-cadherin primers (GenBank accession no. NM _004360) sense 5'-CAC AGG AGT CAT CAG TGT GGT CAC- 3', antisense 5' -CAC CTT GAA GGT CAG CAG CTT G- 3' amplify a 92 bp product.

The PCR amplifications were done under the following conditions: 95°C for 15 min followed by: for 18S ribosomal RNA: 94°C for 45 sec, 60°C for 30 sec, 72°C for 30 sec for 5 cycles followed by 15 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec and a single cycle of 10 min at 72°C and 10 min at 10°C. The same program was used for p15 except the initial annealing temperature was 62°C followed by 35 cycles with annealing temperature of 61°C. A similar program was used for p73 except the initial annealing temperature was 62°C followed by 35 cycles with annealing temperature of 60°C. For each gene, the number of cycles was terminated during the exponential phase of DNA amplification. The PCR products were electrophoresed on 2% agarose gel and detected by ethidium bromide staining. For quantitative detection of gene expression we used 18S ribosomal RNA as the internal standard. The amount of cDNA to use as template for each gene was determined so that it would amplify the identical amount of 18S ribosomal RNA (±20%) during exponential phase. We measured the absolute concentration of amplified DNA by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). This instrument determines the size and amount of DNA using capillary electrophoresis and detection by fluorescence with high precision. The relative amount of amplified DNA for each specific gene to the amount of amplified 18S RNA DNA was quantified for each sample.

Statistical analysis. For each of the genes the mean value for each dose level was obtained and analyzed for variance. To quantitate the difference between mean values, we used the multiple comparison method with the SIDAK correction.

Results

Antineoplastic effect of 5AZA. The effects of different concentrations of 5AZA on the inhibition of growth in KG1a and HL-60 leukemic cells after 72-h exposure are shown in Table I. The effect of different concentrations of 5AZA on inhibition of colony formation was evaluated by clonogenic assay as is also shown in Table I. For both leukemic cell lines the inhibition of growth and colony formation increased with the concentration of 5AZA. At the concentration of 50 ng/ml of 5AZA we demonstrated a loss of clonogenicity of 100% for HL-60 cells. The effect of different concentrations of 5AZA on the inhibition of DNA synthesis in KG1a and Hl-60 cells is shown in Table II. For both leukemic cell lines the inhibition of DNA synthesis increased with the concentration of 5AZA.

Effect of 5AZA on gene expression. The effect of different concentrations of 5AZA on p15 gene expression in KG1a myeloid leukemic cells is shown in Figure 1. The expression of p15 increased with the concentrations of 5AZA as shown by agarose gel stained with ethidium bromide and quantitation of amplified DNA by Bioanalyzer analysis. The greatest expression of p15 was observed with 100 ng/ml of 5AZA, but significant expression of p15 was also obtained with 5 ng/ml of 5AZA. DNA methylation of p15 has been reported previously for KG1a leukemic cells (12).

In our analysis of p73 expression in KG1a cells, we only detected gene re-activation at 100 ng/ml of 5AZA (Figure 2). Lower concentrations of this analog did not re-activate the gene. For E-cadherin expression in HL-60 leukemic cells we detected some base line expression without drug treatment (Figure 3). Only at 100 ng/ml of 5AZA did we detect a large increase in expression of E-cadherin (almost 9-fold).

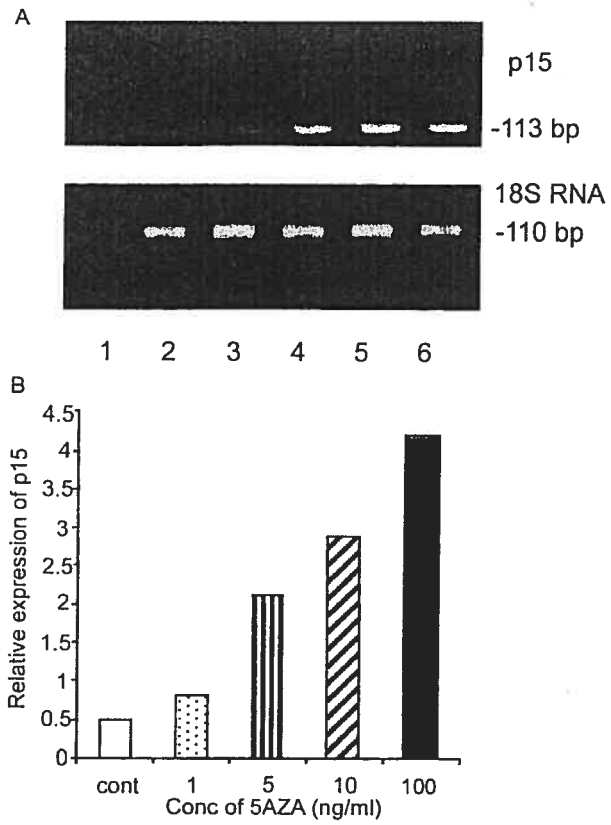

Figure 1. *Effect of different concentrations of 5AZA on the activation of p15 expression in KG1a myeloid leukemic cells. The cells were treated with the indicated concentrations of 5AZA. Total RNA was isolated at 72h for RT-PCR analysis by ethidium bromide staining agarose gel (A). 18S ribosomal RNA was used as the reference standard. 1, water; 2, no treatment; 3, 5AZA 1 ng/ml; 4, 5AZA 5 ng/ml; 5, 5AZA 10 ng/ml; 6, 5AZA 100 ng/ml. The amount of p15 DNA amplified was also determined by Agilent Bioanalyzer (B).*

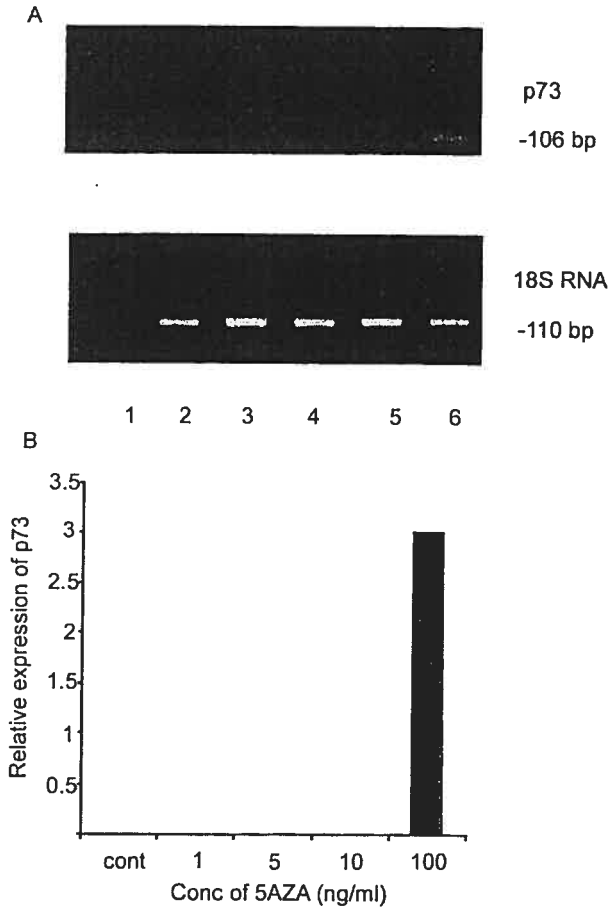

Figure 2. *Effect of different concentrations of 5AZA on the activation of p73 expression in KG1a myeloid leukemic cells. The cells were treated with the indicated concentrations of 5AZA. Total RNA was isolated at 72h for RT-PCR analysis by ethidium bromide staining agarose gel (A). 18S ribosomal RNA was used as the reference standard. 1, water; 2, no treatment; 3, 5AZA 1 ng/ml; 4, 5AZA 5 ng/ml; 5, 5AZA 10 ng/ml; 6, 5AZA 100 ng/ml. The amount of p73 DNA amplified was also determined by Agilent Bioanalyzer (B).*

Discussion

The re-activation of genes that suppress neoplasia by the potent inhibitor of DNA methylation, 5AZA, a cytosine nucleoside analog, is a novel mechanism of action. Optimization of its administration will help realize its full potential since the antineoplastic activity of 5AZA is very dose-schedule-dependent due to its S-phase specificity and short *in vivo* half-life (6). In addition, therapy with 5AZA can produce severe hematopoietic toxicity, its major side-effect (13).

The objective of this study was to find the *in vitro* concentrations that produce a potent antineoplastic effect on human leukemic cell lines and to correlate these data with the concentrations required to re-activate genes that suppress leukemogenesis. Using 5AZA in the range of 1 to 100 ng/ml, we observed that both the inhibition of growth, DNA synthesis and colony formation increased with the concentration of this analog (Tables I and II). It is interesting to note that 100 ng/ml 5AZA, which produced a 100% loss of clonogenicity for HL-60 leukemic cells, also leads to the most pronounced reactivation of the expression of p15, p73 and E-cadherin. (Figures 1-3). This correlation suggests that one mechanism by which 5AZA produces an irreversible loss of clonogenicity is related to its re-activation of genes that suppress leukemogenesis. We do not know which are the most important genes involved in this process. It is possible that the re-activation of other genes that were not investigated in this study are also required to block colony formation by leukemic cells.

In an attempt to translate these data into clinical application, it is tempting to speculate that the minimal plasma concentration of 5AZA should be in the range of 100 ng/ml. In clinical trials on patients with leukemia, remissions were observed when 5AZA was infused at a rate of 30 mg/m^2/h, which produced plasma levels in the range of 200

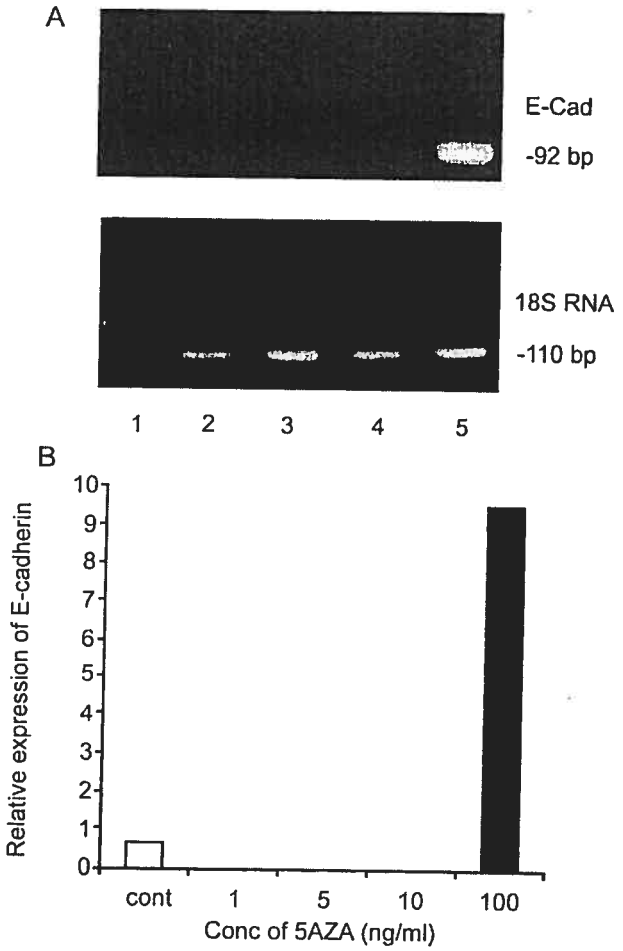

Figure 3. *Effect of different concentrations of 5AZA on the activation of E-cadherin expression in HL-60 myeloid leukemic cells. The cells were treated with the indicated concentrations of 5AZA. Total RNA was isolated at 72 h for RT-PCR analysis by ethidium bromide staining agarose gel (A). 18S ribosomal RNA was used as the reference standard. 1, water; 2, no treatment; 3, 5AZA 1 ng/ml; 4, 5AZA 5 ng/ml; 5, 5AZA 10 ng/ml; 6, 5AZA 100 ng/ml. The amount of E-cadherin DNA amplified was also determined by Agilent Bioanalyzer (B). E-cad, E-cadherin.*

to 400 ng/ml (6,13). Lower doses of 5AZA were used to treat patients with myelodysplastic syndrome (9). In these patients 5AZA was infused at a rate of 1.67 mg/m^2/h (9), which by pharmacokinetic calculations should have produced a plasma level in the range of 10 ng/ml. This concentration of 5AZA showed significant re-activation of p15 in leukemic cell lines, although to a lesser extent than seen at 100 ng/ml (Figure 1).

The low dose therapy of myelodysplastic syndrome with 5AZA produced demethylation and protein expression of p15 in some patients (14).

Future investigations on the analysis of gene re-activation by 5AZA in patients with leukemia and correlation with its response should also provide data that will assist in the design of the optimal dose-schedule for this analog.

Acknowledgements

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References

1 Jones PA and Laird PW: Cancer epigenetics comes of age. Nature Genetics *21*: 163-167, 1999.

2 Wong IH, Ng MH, Huang DP and Lee JC: Aberrant p15 promoter methylation in adult and childhood acute leukemias of nearly all morphologic subtypes: potential prognostic implications. Blood *95*: 1942-1949, 2000.

3 Kawano S, Miller CW, Gombart AF, Bartram CR, Matsuo Y, Asou H, Sakashita A, Said J, Tatsumi E and Koeffler HP: Loss of p73 gene expression in leukemias/lymphomas due to hypermethylation. Blood *94*: 1113-1120, 1999.

4 Melki JR, Vincent PC, Brown RD and Clark SJ: Hypermethylation of E-cadherin in leukemia. Blood *95*: 3208-3213, 2000.

5 Momparler RL and Bovenzi V: DNA methylation and cancer. J Cell Physiol *183*: 145-154, 2000.

6 Momparler RL: Molecular, cellular and animal pharmacology of 5-aza-2'-deoxycytidine. Pharmacol Ther *30*: 287-299, 1985.

7 Kantarjian HM, O'Brien SM, Keating M, Beran M, Estey E, Giralt S, Kornblau S, Rios MB, de Vos D and Talpaz M: Results of decitabine therapy in the accelerated and blastic phases of chronic myelogenous leukemia. Leukemia *11*: 1617-1620, 1997.

8 Zagonel V, Lo Re G, Marotta G, Babare R, Sardeo G, Gattei V, De Angelis V, Monfardini S and Pinto A: 5-Aza-2'-deoxycytidine (Decitabine) induces trilineage response in unfavorable myelodysplastic syndromes. Leukemia *7*: 30-35, 1993.

9 Wijermans P, Lubbert M, Verhoef G, Bosly A, Ravoet C, Andre M and Ferrant A: Low-dose 5-aza-2'-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J Clin Oncol *18*: 956-962, 2000.

10 Momparler RL, Côté S and Eliopoulos N: Pharmacological approach for optimization of the dose schedule of 5-Aza-2'-deoxycytidine (Decitabine) for therapy of leukemia. Leukemia *11*: 175-180, 1997.

11 Primeau M, Gagnon J and Momparler RL: Synergistic antineoplastic action of DNA methylation inhibitor 5-AZA-2'-deoxycytidine and histone deacetylase inhibitor depsipeptide on human breast carcinoma cells. Int J Cancer *103*: 177-184, 2003.

12 Dodge JE, Munson C and List AF: KG-1 and KG-1a model the p15 CpG island methylation observed in acute myeloid leukemia patients. Leuk Res *25*: 917-925, 2001.

13 Rivard GE, Momparler RL, Demers J, Benoit P, Raymond R, Lin KT and Momparler LF: Phase I study on 5-aza-2'-deoxycytidine in children with acute leukemia. Leuk Res *5*: 453-462, 1981.

14 Daskalakis M, Nguyen TT, Nguyen C, Guldberg P, Kohler G, Wjermans P, Jones PA and Lubbert M: Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2'-deoxycytidine (decitabine) treatment. Blood *100*: 2957-2964, 2002.

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CHAPTER XV: OTHER RESULTS

15.1. Accuracy of the Agilent Bioanalyser and of the Method

To test the method, the PCR for a known amount of p15 was repeated 3 times on 4 different amounts of p15 molecules (10^4, 10^5, 10^6, 10^7 molecules) to obtain a standard curve (see figure 15.1 and figure 15.2). The r^2 coefficient of the relation between molecules of p15 and concentration after amplification is 0,96. This regression analysis shows indirectly the accuracy of the method.

A similar experiment, where the PCR was done only once for each concentration but where we repeated 3 times the Agilent Bioanalyzer measurement, showed a r^2 of 0.99.

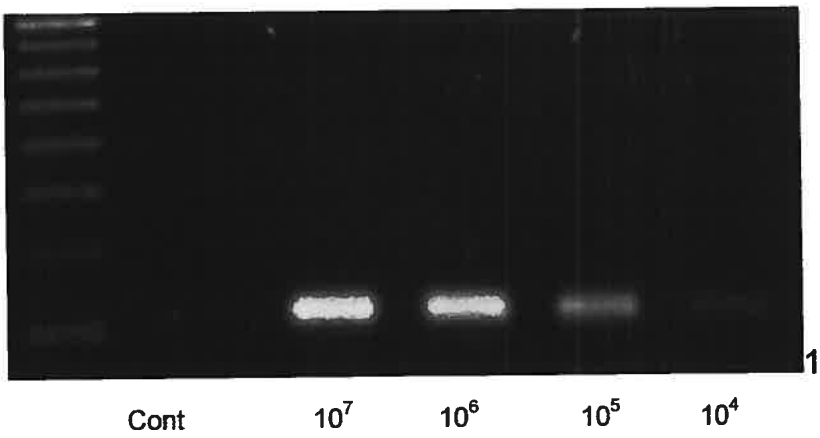


Figure 15.1: Electrophoresis of the p15 standard curve. PCR reaction for p15. The PCR were performed with the same conditions as those used in the study of this candidate gene but on a known amount of molecules of p15. A sample of p15 of our experiment was used. By Bionalyzser we measured the concentrations of p15 and we calculated the number of molecules which were then further diluted.

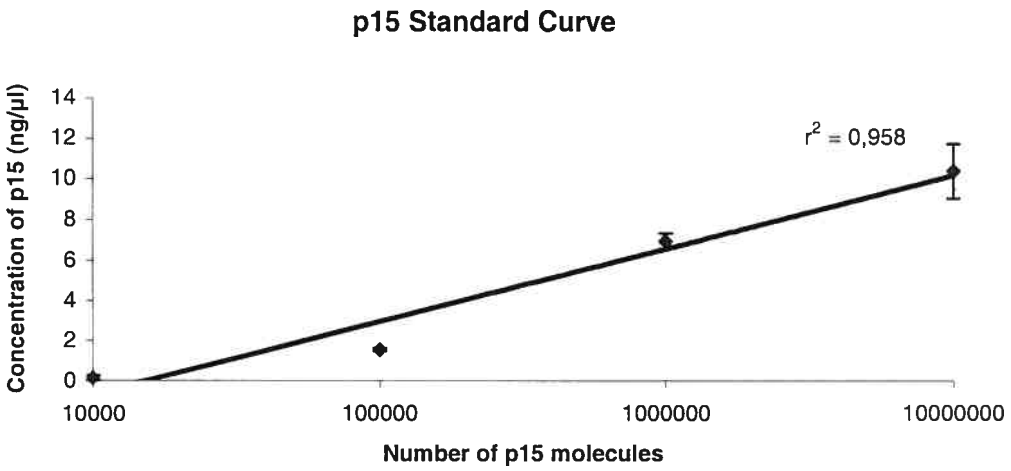


Figure 15.2: P15 standard curve. From each of the 3 PCR reactions done the concentration of p15 was measured by Bionalyzer and put on a logarithmic curve. The results are mean value ± SD. A log trendline is shown.

15.2. Gene Re-expression

Gene expression is described in the manuscript.

15.2.1 p73 Gene expression

For p73 using different primers (identified as primer 2) we obtain some expression of p73 at the concentration of 10 ng/ml. That is shown in figure 15.3. For all the other genes no difference was observed when testing different primers including when we used β-2-microglobulin instead of 18S ribosomal RNA.

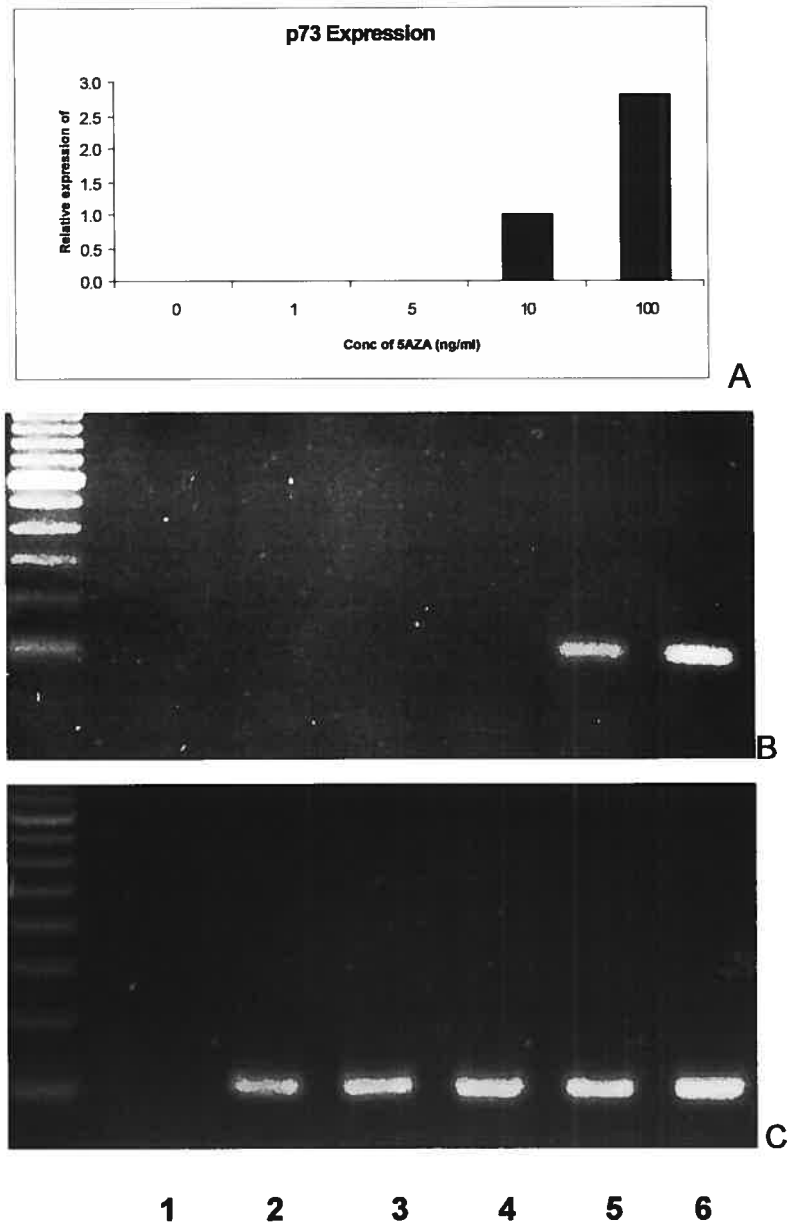


Fig 15.3 Effect of different concentrations of 5AZA on the activation of p73 expression in KG1a using a second set of primers. The cells were treated with the indicated concentrations of 5AZA: 1, water; 2, no treatment; 3, 5AZA 1 ng/ml; 4, 5AZA 5 ng/ml; 5, 5AZA 10 ng/ml; 6, 5AZA 100 ng/ml. Total RNA was isolated at 72h for RT-PCR analysis by ethidium bromide staining agarose gel (B). 18S ribosomal RNA was used as the reference standard C. The amplified gene was measured by Agilent Bioanalyzer (A).

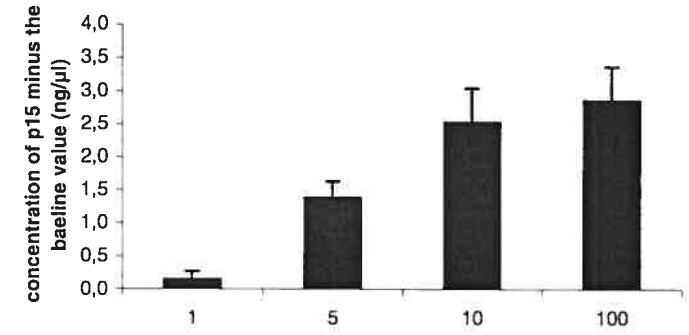
15.2.2 Different Ways of Presenting the Re-expression of p15

In the manuscript, are presented the absolute values obtained in ng/µl. These values were obtained with approximately the same amount of 18S in each sample allowing us to assume the same amount of cDNA.

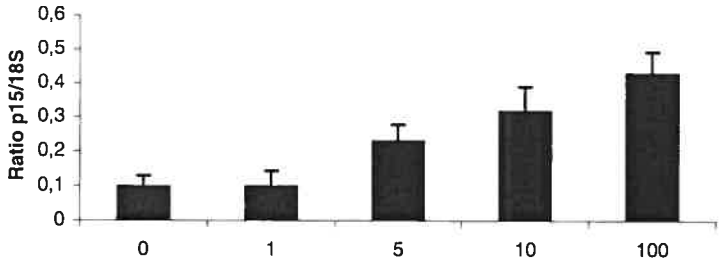
The analyses of variance of the different doses of 5AZA on p15 gene expression showed a global effect ($p \leq 0.001$). When we look at the absolute values of p15, there is an increase in p15 expression beginning at the concentration of 1 ng/ml and statistically significant between 1 and 10 ($p \leq 0.004$). Between 10 and 100 ng/ml, 5AZA it keeps on increasing but without statistical significance. The tendency analysis showed a linear increase starting at 1 ng/ml until 100 ng/ml.

If we subtract the baseline value from the other values, the same effect is seen (figure 15.4A).

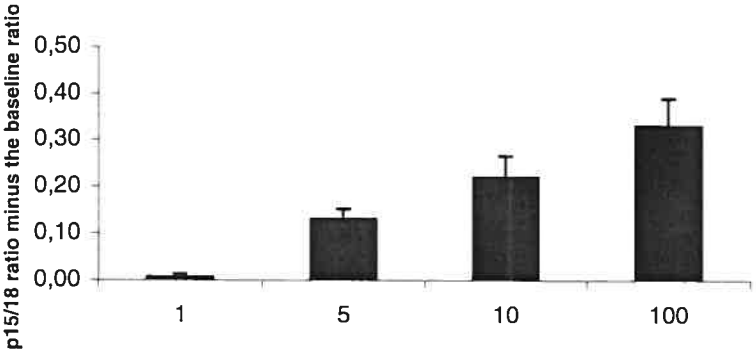
We could also present the ratio of p15 gene by the 18S ribosomal gene value (figure 15.4B) or subtract from this ratio the baseline value (figure 15.4C). In both cases, the global result always remains very significant ($p<0.001$) and the major increase remains between 1 ng/ml and 10 ng/ml.



A



B



C

Fig 15.4: Effect of different concentrations of 5AZA on the activation of p15 gene expression in KG1a cells. The data is presented subtracting the baseline value from the other values (A), reporting the ratio of the p15 gene concentration to the 18S ribosomal gene concentration (ratio p15/18S) (B) and subtracting the baseline ratio to this ratio (15/18S minus the baseline ratio).

15.3 Cell Morphology and Differentiation

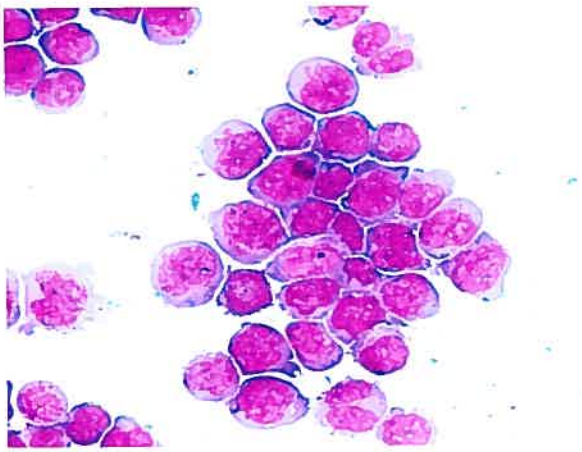
15.3.1 Microscopy

HL-60 cells are shown in fig 15.5A and KG1a cells are shown in fig 15.5B, using a Giemsa Wright stain. HL-60 cells are cells with high nucleocytoplasmic ratio. The cytoplasm is basophilic. The nucleus has loose chromatin and some nucleoli. They tend to aggregate and sometimes form a syncytium (figure 15.5A). The peroxydase stain is negative.

KG1a cells are large irregular cells with high nucleocytoplasmic ratio. Some are multinucleated and have several nuclei (figure 15.5B). The peroxydase stain is negative.

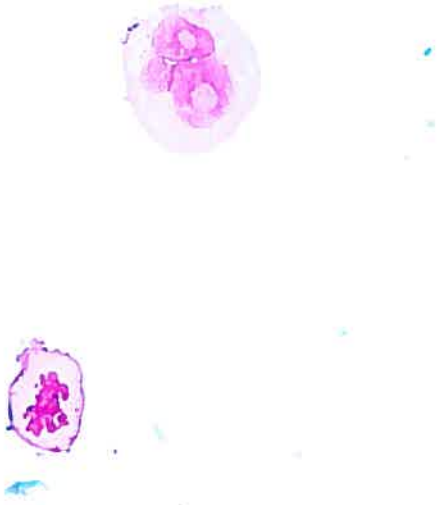
We observed at several time points, the leukemic cells following Giemsa Wright staining: at the beginning of treatment and at 72 h. At 72 h we did not observe more differentiated cells like myelocytes, metamyelocytes, bands or neutrophils or signs of apoptosis. Nor have we observed other types of cells.

HL-60 cells



A

KG1a cells



B

Fig 15.5 Giemsa Wright staining of KG1a (B) and HL-60 (A) cells at 40x magnification.

15.3.2 Immunophenotyping

During one experiment the cells were phenotyped with anti-sera which usually mark AML: once at the beginning and once at 72 hours following the beginning of treatment with anti-sera for CD13, CD 33, CD7, CD15, CD11

Before treatment:

KG1a cells were CD13, CD33, CD7, CD15 positive.

HL-60 cells were CD13 and CD33, positive.

At 72 h:

KG1a cells were CD33, CD7, CD15, CD13, CD11a positive. CD11b negative.

HL-60 cells are CD33, CD13 and CD11a positive CD11b negative

15.4. Real-Time PCR

Real-Time PCR was attempted several time for p15. First a standard curve was performed which is shown on figure 15.6. But when run with the p15 gene, we obtained several primer dimers which did not allow interpretation. This problem could not be overcome with any of the sets of the primers.

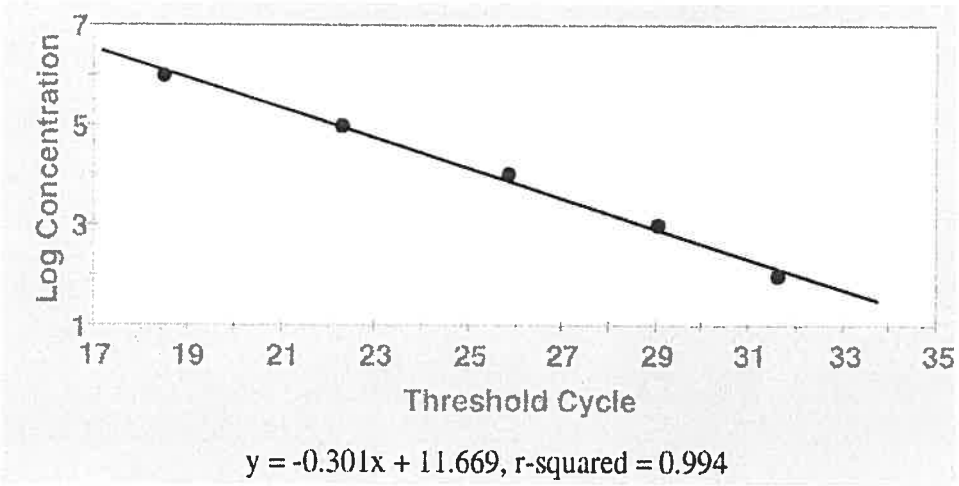


Fig 15.6: The standard curve for p15 using Real Time PCR. In this curve there are only shown the results of one experiment. The standard curve was repeated several times by other researchers in the laboratory.

PART V: DISCUSSION

CHAPTER XVI: DISCUSSION

5AZA has a novel mechanism of action of reactivating silent TSGs by demethylation. This action can lead to loss of clonogenicity of leukemic cells due to induction of terminal differentiation or senescence. 5AZA is an S phase specific agent with a short in vivo half-life due to rapid inactivation by cytidine deaminase. All these parameters should be taken into consideration in the design of the optimal dose-schedule of 5AZA for the curative therapy of leukemia.

Gene Re-activation

In this study 5AZA was shown to re-activate the TSGs, p15, p73 and E-cadherin in human myeloid leukemic cell lines and produced a significant antileukemic effect that confirmed our hypothesis. It was possible to detect reactivation of p15 at a concentration of 5 ng/ml of 5AZA, whereas reactivation of p73 was only at 10 ng/ml and of E-cadherin at 100 ng/ml of 5AZA. The reason for this difference in sensitivity is unknown. It is possible that it may involve the binding of different proteins to methylated CpGs in the promoter region.

The reactivation of p15 by 1 ng/ml of 5AZA was more difficult to assess and depends on the way we look at our data. If we look at the mean plots of the results in terms of the absolute value of DNA (ng/µl), there seems to be some reactivation but if we determine a ratio between p15 and 18s the expression at 1 ng/ml disappears. No matter the way we look into our data the major increase of gene reactivation of p15 occurs between 1 ng/ml and 10 ng/ml ($p<0.01$). The increase between 0 or 1 and 100 ng/ml was positive in all cases ($p< 0.001$) and 100 ng/ml produced significant reactivation of all the genes studied. Further studies should be undertaken to see which way of presenting gene re-

expression data would be most precise, using the absolute value of DNA, the value relative to 18S expression or subtracting the baseline value.

We found some baseline gene expression of p15 and E-Cadherin in a cell line supposed to be completely silenced. Hypermethylation of the CpG islands is a highly heterogeneous and variable event[11,68,69] Nevertheless the amount of expression is so small that it could be considered in the range of the background signal of the instrument and not due to real expression.

In this study we assumed that p15, p73 and E-Cadherin were completely silenced by methylation as reported by other authors[21,22,24]. Techniques like DNA sequencing, microsatellite analysis and Southern blotting are needed to show that the gene sequence is normal. The silencing is due to an epigenetic phenomenon which can be demonstrated by bisulfite-DNA sequencing and methylation specific PCR. Sodium bisulfite deaminates cytosine to uracil, whereas 5-methylcytosine is resistant to deamination[70]. Using methylation specific primers, methylation can be identified by PCR. For p15 in KG1a, Dodge et al[22] used sodium bisulfite DNA sequencing. They also looked for gene expression of p15 by RT-PCR and Western blotting. They showed that there was no deletion or mutation in the p15 gene and it was highly methylated, with more than 80% of the CpG sites methylated. In normal bone marrow the p15 gene was unmethylated and expressed. Corn et al[23], investigated the p73 gene in the KG1a cell line. Using Southern blot analysis, PCR in single strand conformational polymorphism they didn't observe any abnormality in the sequence of the p73 gene. They also showed by Southern blotting and methylation specific PCR (MSP) that the gene was methylated. In another study, Corn et al[24] studied the HL-60 cell line looking for alterations in the E-Cadherin gene and gene expression. Genomic bisulfite sequencing showed normal alleles, and full methylation. RNA transcription and protein expression were absent. Peripheral blood mononuclear cells, used as control, were almost unmethylated. Furthermore, in these 3 studies a re-expression of the different genes was shown after treatment with 5AZA. In our laboratory using MSP, it was confirmed that these genes were methylated (unpublished data).

Translation of data on in vitro gene activation to the optimal plasma concentration of 5AZA for therapy of leukemia should be done with caution. The reactivation of one or more TSGs by 5AZA does not necessarily result in a loss of clonogenicity, the primary objective of the chemotherapy. Data on clonogenic assays and pharmacokinetic analysis are more suitable for translation to plasma concentration of 5AZA.

Pharmacokinetics

Pharmacokinetic and pharmacodynamic parameters should be taken into consideration for the design of the optimal dose-schedule of 5AZA. 5AZA has a very short half live of 15-20 minutes. It is mainly eliminated by deamination. Cytidine deaminase is present in high levels in the liver and spleen[71] and in lesser amounts elsewhere in the body. Those two organs are particularly important due to leukemic cell infiltration. To obtain therapeutic concentrations in liver and spleen, a higher dose is required, or alternatively an inhibitor of cytidine deaminase could be given (see below). In leukemia, 5AZA may penetrate less in packed marrow but crosses the blood-brain barrier well with CSF/plasma ratios in the range of 0.25-0.5 in animal models[36]. In a phase I/II clinical trial in children, Rivard et al[39] showed that leukemic cells in the CSF could be cleared by high doses of 5AZA.

In order to be active, 5AZA must be phosphorylated by deoxycytidine kinase, the rate-liming enzyme of the metabolic cascade of 5AZA. The concentration of 5AZA at which this enzymatic reaction takes place at 50% of its maximum velocity (Km) is in the range of 10 μM (2,280 ng/ml)[59]. The optimal plasma concentration for 5AZA would probably be in the range of this limiting Km to obtain close to maximal activation of this analog. However such a concentration would carry unacceptable toxicity unless a short infusion of 5AZA is used.

A key point to consider is that the concentration of 5AZA in all anatomic compartments during therapy will be different. If the concentration in any anatomic compartment is below the minimal cytotoxic level, it will act as a

sanctuary for the leukemic cells. The choice of the dose of 5AZA must take this into consideration.

Cell Kinetics Considerations

5AZA acts in S Phase of the cell cycle and therefore is schedule dependent. So the maximal tolerated individual dose given as a bolus dose might not be the most important parameter[72]. Raza et al[73] estimated that the AML cell cycle length was around 60 h (range 18-211). Only 6 of the 54 patients he studied had cycles longer that 90 h. More recently this group provided additional data[74] in 128 patients with AML who had median cycle times of 45 h (range 11.5 – 211) with a median S phase time was 12 h (range 3.1 –35). Ideally, the duration of infusion of 5AZA should be long enough to permit all of the clonogenic leukemic cells to enter the S phase.

Clonogenic Assays

A clonogenic assay measures the potential of leukemic cells to form colonies in vitro. Since the goal of the chemotherapy is to destroy the proliferative potential of leukemic cells, the concentrations of 5AZA and duration of treatment that completely block colony formation are of key importance. These data are difficult to obtain with leukemic cells from patients due to the large variability in their in vitro growth characteristics. Different human leukemic cell lines can be used for this purpose.

In this study the concentration of 5AZA for a 72 h exposure that produced about 50% loss in clonogenicity was in the range of 10 ng/ml for HL-60 myeloid leukemic cells. At 50 ng/ml, for 72 h, 5AZA produced a 100% loss of clonogenicity for this cell line.

In studies on patients the concentration of 5AZA to obtain 50% cell death (IC_{50}) of leukemic cells from non-resistant AML patients is quite high, around 1 μM (228 ng/ml). In a study by Gattei et al on clonogenic cells from patients with AML the lowest IC_{50} was 0.43 μM (98 ng/ml)[38]. This high IC_{50} will change if we use intermittent dosing providing more prolonged exposure[75].

Prior Clinical Studies on 5AZA

5AZA is now in phase III trials for MDS where small doses are used: 45 mg/m^2/day for 3 days every 6 weeks with an overall response rate of 49%[47]. In this clinical setting Issa et al[41] showed an objective response in more than half of the patients with doses as small as 5 to 20 mg/m^2 given as one-hour infusion, 5 days a week for 2 weeks.

In CML, 5AZA at 75-100 mg/m^2 over a six-hour infusion every 12 h for 5 days showed objective responses in half of patients in accelerated phase and in one quarter of the patients with blastic phase[46].

AML was one of the first pathologies in which 5AZA was studied. Rivard et al[39] in a phase I/II trial entered 15 patients with AML showing a response in a majority of them. They used doses up to 80 mg/Kg and showed that when using 5AZA at 1 mg/kg/h (approximately 30 mg/m^2/h) the plasma levels at 24 h were around 500 ng/ml (300-900).

Richel et al[51] in 1991 treated 11 patients in the first relapse of AML with 5AZA at 125-250 mg/m^2 twice daily as six-hour infusions associated with amsacrine showing 8 CR[51]. He confirmed the cross resistance between ARA-C and 5AZA previously described.

Petti et al[53] and Gattei et al[38] tested 5 AZA in AML using doses of 90-120 mg/m^2 as a single agent administered as a four-hours infusion showing overall responses in more than 1/3 of the patients. Except for myelosuppression the toxicity was mild. Petti recommended 5AZA as a component of therapy for patients with poor general condition or advanced age.

5AZA was used in relapsed AML associated with either amsacrine or idarubicin in a study that also included ALL and CML[54]. 5AZA was given as a six-hour infusion every 12 h for 6 days. 57 patients with relapsed AML entered the study. Among those, 22 obtained a CR after 1 or 2 cycles. Only 20% of the patients were in remission after 1 year. Hematologic and gastrointestinal toxicity were

the most frequently seen. As a first line treatment in AML associated with daunorubicin 50 mg/m^2 day on day 1 to 3, it induced a CR in the 6 evaluable patients[52]. 5AZA was given as a four-hours intravenous infusion of 90 mg/m^2 daily for 5 days. A maximum of two cycles was administered. Myelosuppression and mucositis were the major toxicities observed.

In AML, 5AZA at intermediate/high dose, given as a four-six-hours infusion is active. The studies in which it was combined with other drugs entered small numbers of patients and are too heterogeneous to allow conclusions. Nevertheless the results seem as good as those obtained with classical regimens.

Integration of Data for Translation into Clinical Trials

The recommended dose depends on the endpoint: demethylation, cytotoxicity or survival. Methylation of the TSGs seems to be important in the physiopathology of cancer and so demethylation could be a step to the cure. To date, it is not clear which genes need to be demethylated for the cure of a patient. Most important for the patient is long-term survival with limited side effects. Studies need to be designed to see if re-expression of a specific gene might be used as a surrogate marker for cure in pathologies where epigenetics show a major role, among others, myeloblastic leukemia. Further, we do not know the exact amount of demethylation needed to have a real effect in the clinic. For certain authors[11,68], the density of methylation within the CpG island is the paramount factor of silencing and not methylation of any specific site. Cameron et al[68] showed in vitro that methylation of CpG 40% in each allele is necessary for silencing the gene but this is controversial. Full demethylation of a gene may not be required to have real clinical effects.

If we look for demethylation, the therapeutic plasma levels are less than those previously recommended of 1 μM[31]. Aparicio et al[42] used 5AZA 30 mg/m^2 given over 72 h and obtained plasma levels of 0.12 μM (27 ng/ml) and 0,16 μM (36 ng/ml). They observed demethylation but no objective clinical response in patients with cancer.

We did not observe any morphological signs of differentiation or apoptosis but we only followed the cells for 3 days. We did not use any specific marker of apoptosis like annexin or any marker of differentiation. This was not the objective of the study. We phenotyped the cells before treatment and after 3 days. This was done to see on which cells we had had an effect and was not repeated. At 72 h there were neutrophil markers in both cell lines. This requires confirmation in other studies. It would be interesting to see if there is any correlation between an early appearance of neutrophil markers and gene expression.

Translation into the clinic is particularly difficult when we use cell lines. Mimicking pharmacokinetics in vitro is difficult and uncertain particularly given interpatient variability. The human myeloid leukemia cell lines HL-60 and KG1a do not have the characteristics of differentiated human myeloid cells: different morphology, different staining characteristics and a tendency to aggregate and eventually form syncytia. Nevertheless, morphology is becoming less important in the classification and definition of cancer, with an increasing role of genetics[76]. The leukemia cells studied have been cultured in vitro for years and might have acquired different characteristics. On the other hand, compared to leukemic cells from the patient, they are homogenous and well studied. Studying cells from a patient might allow the selection of different clones and contamination from other human cells. This raises the problem of a good model which has to be homogenous and reproducible and representative of the studied cancer. The techniques used might also lead us into wrong translation. In this study two different primers used to study p73 gave slightly different results with expression of the gene starting at two different concentrations.

Pharmacodiagnostics

Pharmacodiagnostics have an increasingly important role in the choice of the drug and dosage. Clonogenic assays have been shown to be good surrogates for in vivo effect. They are more specific than DNA synthesis assays which are easily performed, although a good relationship between in vitro drug resistance

and a poor clinical response to therapy has been seen using incorporation of thymidine[77]. Today, there is an increasing number of new agents coming to market. In some clinical settings, such as perhaps, non-response to a standard drug regimen these tests can be used to select the best second line drug. They can be associated with others like gene re-expression or the study of certain polymorphisms[78] to try to get a model to predict a response. It is fundamental to improve our models to allow a better correlation between the preclinical and clinical situations[79].

Future Studies

Resistance to drugs is a feared event. Therefore cancer is treated with multidrug regimens in order to overcome resistance and to increase efficacy. Usually, regimens have drugs with different mechanisms of action. For 5AZA there is a clear potentiating effect of the histone deacetylase inhibitors[80,81]. They should be tested in combination and studied in such a way as to ascertain if the acetylation/methylation status correlates with survival.

Zebularine is an interesting medication to test in association with 5AZA, in cellular and animal models. It inhibits cytidine deaminase[82] and might increase the levels of 5AZA in liver and spleen.

Some drugs used mainly in solid tumors have shown some synergy when used with 5AZA such as cisplatin[83,84] and topotecan[85]. A phase II study has been completed with cisplatin and 5AZA, showing a moderate effect on the advanced squamous cell carcinoma of the cervix[83].

In leukemia, there is an interesting synergy described with retinoids and interferons[86] which might be used in myeloblastic leukemia, perhaps particularly promyelocytic leukemia.

Growth factors have also been recommended, myelosuppression being the major limiting toxicity mainly at high doses where it has major cytotoxic effects[31]. We could also test growth factors during treatment with 5AZA to

recruit cells into S phase and increase the activity of 5AZA[87]. This is controversial and would need strong animal model support before being tested in clinic.

A window trial with 5AZA might be interesting to show the effect of this drug as single agent.

Introducing the drug into a therapeutic trial in patients with newly diagnosed myeloblastic leukemia raises two major concerns: a possible decrease in currently achieved results and unknown long term side effect. We now cure 50% of AML in children and more than 30% in adults. A change of protocol could worsen those results. Given the data already obtained on the drug, including better cytotoxic activity than ARA-C with, in addition another mechanism of action, namely demethylation, a pilot randomized study could be considered in newly diagnosed patients after a pilot study in patients with recurrent myeloid leukemia demonstrated safety and preliminary evidence of efficacy of the proposed regimen.

No study has investigated the long term side effects of 5AZA. Methylation plays an important role in embyogenesis and fetal development. The effect on the hemoglobin genes is very well known. Some concerns are therefore evident. One of the most feared long-term side effects of chemotherapy is secondary malignancies. Even if the dominant epigenetic effect of 5AZA appears to be demethylation of TSG[88], 5AZA could possibly activate silenced oncogenes. It has already been shown for the HOX1 gene[89]. In AML reactivation or upregulation of oncogenes could potentiate the effects of the epidophylotoxins and alkylating agents. Clinical studies have to include monitoring for this.

Dose of 5AZA

We observed an effect on re-expression of p15 gene from a concentration of 5AZA of 5 ng/ml but we needed 100 ng/ml for p73 and E-Cadherin. For clinical studies, depending on the endpoint defined, we could start with a low or a higher dose of 5AZA. It is not known which gene needs to be demethylate to

have an effect, nor the minimal demethylation needed for the effect. A low dose giving levels around 10 ng/ml could be clinically sufficient. Nevertheless when we look at our data, the concentrations of 100 ng/ml gave for all 3 genes an evident re-expression, which parallels growth inhibition, inhibition of the DNA synthesis, and loss of clonogenicity. So we could foresee that a dose, which would give levels round 100 ng/ml, would have more clinical effects. Furthermore, the cells were only treated once for KG1a and once daily for HL-60. So we have an argument to test in clinical trials high dose boluses of 5AZA. Nevertheless the doubling time of the HL-60 and KG1a may be less than certain human leukemic clones and the drug in vitro is not destroyed by deamination and has a longer half live. Finally increasing the dose in clinical trials might induce the re-expression of certain genes, which could carry unwanted effects.

Ethical Considerations

In this study, we were working on a cell model from an anonymized patient so very few ethical problems were raised. Anonymization of the cells had already been performed.

Examining the history of 5AZA there are 2 major ethical points worth noting: The first one concerns the publication of negative results[44] fundamental when we investigate new drugs. The international tendency of not publishing negative results might give clinicians an erroneous view of the drug. The second one concerns phase I trials in children. For 5AZA, the first phase I trial was published in pediatrics[39].

Using children as research subjects, is a very controversial issue[90] mainly when it comes to phase I trials. Research is essential for improving cure rates and quality of life. It is not ethical that any social group should be deprived of its benefits and so, the American Academy of Pediatrics recommends that children should benefit from research including from phase I trials. Data obtained from adult research is not always valid in pediatrics.

Children are a very vulnerable population and should be protected from research abuse. The Nuremberg code, the Helsinki convention and the constitution of many countries state that no harm is acceptable to children simply to benefit others. Medications evaluated in phase I studies have a small but real possible therapeutic benefit to offer children with advanced malignant disease. The overall response rates in phase I studies, depending on the disease, range from <3% to 17.7%, with a toxic mortality rate of less than 1%[91]. Response may carry clinical benefit, but not in all cases. The overall impact on quality of life is uncertain. Eligibility criteria do require that the child be sufficiently fit physiologically to receive therapy.

Autonomy is also a problem; parents decide on the child's behalf, particularly in cases in which the child is less than 10 years of age. In this setting, parents

may also have unrealistic hopes. Many patients and families seek enrolment on a phase I study with the expectation of potential improvement[92]. Physicians may also expect an overall benefit to the child from enrolment on a phase I study[93,94]. An inquiry from the Children`s Oncology Group and the United Kingdom Children`s Cancer Study Group showed that the majority considered that there was benefit to the child from participation in a phase I study[95]. Those benefits may include psychological benefit, hope and altruism. A phase I study should interfere as little as possible with the child's quality of life. Also, the patient, if old enough, and family must understand the limited possibility of benefit.

In phase II and III trials this controversy is less intense due to the possible benefits that might be anticipated. Currently, given the large number of drugs being tested an important issue is the choice of which to introduce in the clinic. Concerning 5AZA, it has shown promising activity and so further studies seem indicated.

Final Considerations

In conclusion, there are not enough data to incorporate 5AZA into a rationally designed combination regimen. If we accept the hypothesis that gene re-expression is important to the cure of leukemia and if we want to test that re-expression as a surrogate marker, our data present an argument to plan such a study starting with doses that give levels around 10 ng/ml eventually in association with histone deacetylase inhibitors.

Further studies in animal models looking for the effect of a intermediate/high dose scheme (plasma levels 100-200 ng/ml) and testing for different times of perfusion would be helpful. This could be performed in association with other drugs used in leukemia and eventually with G-CSF. Similar data should be obtained with low doses (plasma levels 10-50 ng/ml). It would be interesting to know which plasma levels we achieve with the small doses used by Issa[41] and correlate them with gene activation, clonogenicity and clinical effects.

In patients with AML, lower doses of 5AZA could be tested in groups not expected to tolerate high doses. For the other patients a pilot trial could be used to test the intermediate/high doses of 5AZA. If initial studies prove promising, subsequent studies will need to examine late effects as well.

BIBLIOGRAPHY

1. Lowenberg B, Downing JR, Burnett A: Acute myeloid leukemia. N. Engl. J. Med. 341(14): 1051-62, 1999.

2. Jones PA, Laird PW: Cancer epigenetics comes of age. Nat. Genet. 21(2): 163-67, 1999.

3. Momparler RL: Cancer epigenetics. Oncogene 22(42): 6479-83, 2003.

4. Ehrlich M, Gama-Sosa MA, Huang LH, Midgett RM, Kuo KC, McCune RA, Gehrke C: Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. Nucleic. Acids Res. 10: 2709-21, 1982.

5. Razin A, Riggs AD: DNA methylation and gene function. Science 210(4470): 604-10, 1980.

6. Kafri T, Ariel M, Brandeis M, Shemer R, Urven L, McCarrey J, Cedar H, Razin A: Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. Genes Dev. 6(5): 705-14, 1992.

7. Kass SU, Pruss D, Wolffe AP: How does DNA methylation repress transcription? Trends Genet. 13(11): 444-49, 1997.

8. Nephew KP, Huang TH: Epigenetic gene silencing in cancer initiation and progression. Cancer Lett. 190(2): 125-33, 2003.

9. Momparler RL, Bovenzi V: DNA methylation and cancer. J. Cell Physiol. 183(2):145-54, 2000.

10. Herman JG, Civin CI, Issa JP, Collector MI, Sharkis SJ, Baylin SB: Distinct patterns of inactivation of p15INK4B and p16INK4A characterize the major types of hematological malignancies. Cancer Res. 57: 837-41, 1997.

11. Dodge JE, List AF, Futscher BW: Selective variegated methylation of the p15 CpG island in acute myeloid leukemia. Int. J. Cancer 78(5): 561-67, 1998.

12. Claus R, Lubbert M: Epigenetic targets in hematopoietic malignancies. Oncogene. 22(42): 6489-96, 2003.

13. Okuda T, Shurtleff SA, Valentine MB, Raimondi SC, Head DR, Behm F, Curcio-Brint AM, Liu Q, Pui CH, Sherr CJ: Frequent deletion of p16INK4a/MTS1 and p15INK4b/MTS2 in pediatric acute lymphoblastic leukemia. Blood 85(9): 2321-30, 1995.

14. Wong IH, Ng MH, Huang DP, Lee JC: Aberrant p15 promoter methylation in adult and childhood acute leukemias of nearly all morphologic subtypes: potential prognostic implications. Blood 95(6): 1942-9, 2000.

15. Heyman M, Rasool O, Borgonovo BL, Liu Y, Grander D, Soderhall S, Gustavsson G, Einhorn S: Prognostic importance of p15INK4B and p16INK4 gene inactivation in childhood acute lymphocytic leukemia. J. Clin. Oncol. 14(5): 1512-20, 1996.

16. Maloney KW, McGavran L, Odom LF, Hunger SP: Acquisition of p16(INK4A) and p15(INK4B) gene abnormalities between initial diagnosis and relapse in children with acute lymphoblastic leukemia. Blood 93(7): 2380-85, 1999.

17. Chim CS, Tam CY, Liang R, Kwong YL: Methylation of p15 and p16 genes in adult acute leukemia: lack of prognostic significance. Cancer 91(12): 2222-29, 2001.

18. Kawano S, Miller CW, Gombart AF, Bartram CR, Matsuo Y, Asou H, Sakashita A, Said J, Tatsumi E, Koeffler HP: Loss of p73 gene expression in leukemias/lymphomas due to hypermethylation. Blood 94(3):1113-20, 1999.

19. Liu M, Taketani T, Li R, Takita J, Taki T, Yang HW, Kawaguchi H, Ida K, Matsuo Y, Hayashi Y: Loss of p73 gene expression in lymphoid leukemia cell lines is associated with hypermethylation. Leuk. Res. 25(6): 441-47, 2001.

20. Melki JR, Vincent PC, Brown RD, Clark SJ: Hypermethylation of E-cadherin in leukemia. Blood 95(10): 3208-13, 2000.

21. Herman JG, Jen J, Merlo A, Baylin SB: Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B. Cancer Res. 56(4): 722-27, 1996.

22. Dodge JE, Munson C, List AF: KG-1 and KG-1a model the p15 CpG island methylation observed in acute myeloid leukemia patients. Leuk. Res. 25(10): 917-25, 2001.

23. Corn PG, Kuerbitz SJ, van Noesel MM, Esteller M, Compitello N, Baylin SB, Herman JG: Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5' CpG island methylation. Cancer Res. 59(14): 3352-56, 1999.

24. Corn PG, Smith BD, Ruckdeschel ES, Douglas D, Baylin SB, Herman JG: E-cadherin expression is silenced by 5' CpG island methylation in acute leukemia. Clin. Cancer Res. 6(11): 4243-48, 2000.

25. Sobrinho-Simoes M, Oliveira C: Different types of epithelial cadherin alterations play different roles in human carcinogenesis. Adv Anat Pathol 9(6): 329-37, 2002.

26. Nollet F, Berx G, van Roy F: The role of the E-cadherin/catenin adhesion complex in the development and progression of cancer. Mol Cell Biol Res Commun 2(2): 77-85, 1999.

27. Jones PA, Taylor SM: Cellular differentiation, cytidine analogs and DNA methylation. Cell 20(1): 85-93, 1980.

28. Pinto A, Attadia V, Fusco A, Ferrara F, Spada OA, Di Fiore PP: 5-Aza-2'-deoxycytidine induces terminal differentiation of leukemic blasts from patients with acute myeloid leukemias. Blood 64(4): 922-29, 1984.

29. Momparler RL, Bouchard J, Samson J: Induction of differentiation and inhibition of DNA methylation in HL-60 myeloid leukemic cells by 5-AZA-2'-deoxycytidine. Leuk. Res. 9(11): 1361-66, 1985.

30. Onetto N, Momparler RL, Momparler LF, Gyger M: In vitro biochemical tests to evaluate the response to therapy of acute leukemia with cytosine arabinoside or 5-AZA-2'-deoxycytidine. Semin. Oncol. 14(2): 231-37, 1987.

31. Momparler RL, Cote S, Eliopoulos N: Pharmacological approach for optimization of the dose schedule of 5-Aza-2'-deoxycytidine (Decitabine) for the therapy of leukemia. Leukemia 11 Suppl 1: S1-6, 1997.

32. Momparler RL, Bouffard DY, Momparler LF, Dionne J, Belanger K, Ayoub J: Pilot phase I-II study on 5-aza-2'-deoxycytidine (Decitabine) in patients with metastatic lung cancer. Anticancer Drugs 8(4): 358-68, 1997.

33. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB: Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat. Genet. 21(1): 103-7, 1999.

34. Wilson VL, Jones PA, Momparler RL: Inhibition of DNA methylation in L1210 leukemic cells by 5-aza-2'-deoxycytidine as a possible mechanism of chemotherapeutic action. Cancer Res. 43(8): 3493-96, 1983.

35. Momparler RL, Momparler LF, Samson J: Comparison of the antileukemic activity of 5-AZA-2'-deoxycytidine, 1-beta-D-arabinofuranosylcytosine and 5-azacytidine against L1210 leukemia. Leuk. Res. 8(6):1043-49, 1984.

36. Chabot GG, Rivard GE, Momparler RL: Plasma and cerebrospinal fluid pharmacokinetics of 5-Aza-2'-deoxycytidine in rabbits and dogs. Cancer Res. 43(2): 592-97, 1983.

37. Momparler RL, Bouchard J, Onetto N, Rivard GE: 5-aza-2'-deoxycytidine therapy in patients with acute leukemia inhibits DNA methylation. Leuk. Res. 8(2):181-85, 1984.

38. Gattei V, Aldinucci D, Petti MC, Da PA, Zagonel V, Pinto A: In vitro and in vivo effects of 5-aza-2'-deoxycytidine (Decitabine) on clonogenic cells from acute myeloid leukemia patients. Leukemia 7 Suppl 1: 42-48, 1993.

39. Rivard GE, Momparler RL, Demers J, Benoit P, Raymond R, Lin K, Momparler LF: Phase I study on 5-aza-2'-deoxycytidine in children with acute leukemia. Leuk. Res. 5(6): 453-62, 1981.

40. van Groeningen GC, Leyva A, O'Brien AM, Gall HE, Pinedo HM: Phase I and pharmacokinetic study of 5-aza-2'-deoxycytidine (NSC 127716) in cancer patients. Cancer Res. 46(9): 4831-36, 1986.

41. Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J, Kantarjian HM: Phase I study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (Decitabine) in hematopoietic malignancies. Blood 103(5): 1635-40, 2004.

42. Aparicio A, Eads CA, Leong LA, Laird PW, Newman EM, Synold TW, Baker SD, Zhao M, Weber JS: Phase I trial of continuous infusion 5-aza-2'-deoxycytidine. Cancer Chemother. Pharmacol. 51(3): 231-39, 2003.

43. Ravandi F, Kantarjian H, Cohen A, Davis M, O'Brien S, Anderlini P, Andersson B, Claxton D, Donato M, Gajewski J, Khouri I, Korbling M, Ueno N, deVos D, Champlin R, Giralt S: Decitabine with allogeneic peripheral blood stem cell transplantation in the therapy of leukemia relapse following a prior transplant: results of a phase I study. Bone Marrow Transplant. 27(12): 1221-25, 2001.

44. Clavel M, Monfardini S, Fossa S, Smyth J, Renard J, Kaye SB: 5-Aza-2'-deoxycytidine (NSC 127716) in non-seminomatous testicular cancer. Phase II from the EORTC Early Clinical Trials Cooperative Group and Genito-Urinary Group. Ann. Oncol. 3(5): 399-40, 1992.

45. Kantarjian HM, O'Brien SM, Keating M, Beran M, Estey E, Giralt S, Kornblau S, Rios MB, de Vos D, Talpaz M: Results of decitabine therapy in the accelerated and blastic phases of chronic myelogenous leukemia. Leukemia 11(10): 1617-20, 1997.

46. Kantarjian HM, O'Brien S, Cortes J, Giles FJ, Faderl S, Issa JP, Garcia-Manero G, Rios MB, Shan J, Andreeff M, Keating M, Talpaz M: Results of decitabine (5-aza-2'deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. Cancer 98(3): 522-8, 2003.

47. Wijermans P, Lubbert M, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A: Low-dose 5-aza-2'-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J. Clin. Oncol. 18(5): 956-62, 2000.

48. Daskalakis M, Nguyen TT, Nguyen C, Guldberg P, Kohler G, Wijermans P, Jones PA, Lubbert M: Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2'-deoxycytidine (decitabine) treatment. Blood 100(8): 2957-64, 2002.

49. Lubbert M, Wijermans P, Kunzmann R, Verhoef G, Bosly A, Ravoet C, Andre M, Ferrant A: Cytogenetic responses in high-risk myelodysplastic syndrome following low-dose treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine. Br. J. Haematol. 114(2): 349-57, 2001.

50. Wijermans PW, Krulder JW, Huijgens PC, Neve P: Continuous infusion of low-dose 5-Aza-2'-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. Leukemia 11 Suppl 1: S19-23, 1997.

51. Richel DJ, Colly LP, Kluin-Nelemans JC, Willemze R: The antileukaemic activity of 5-Aza-2 deoxycytidine (Aza-dC) in patients with relapsed and resistant leukaemia. Br. J. Cancer 64(1): 144-48, 1991.

52. Schwartsmann G, Fernandes MS, Schaan MD, Moschen M, Gerhardt LM, Di LL, Loitzembauer B, Kalakun L: Decitabine (5-Aza-2'-deoxycytidine; DAC) plus daunorubicin as a first line treatment in patients with acute myeloid leukemia: preliminary observations. Leukemia 11 Suppl 1: S28-31, 1997.

53. Petti MC, Mandelli F, Zagonel V, De GC, Merola MC, Latagliata R, Gattei V, Fazi P, Monfardini S, Pinto A: Pilot study of 5-aza-2'-deoxycytidine (Decitabine) in the treatment of poor prognosis acute myelogenous leukemia patients: preliminary results. Leukemia 7 Suppl 1: 36-41, 1993.

54. Willemze R, Suciu S, Archimbaud E, Muus P, Stryckmans P, Louwagie EA, Berneman Z, Tjean M, Wijermans P, Dohner H, Jehn U, Labar B, Jaksic B, Dardenne M, Zittoun R: A randomized phase II study on the effects of 5-Aza-2'-deoxycytidine combined with either amsacrine or idarubicin in patients with relapsed acute leukemia: an EORTC Leukemia Cooperative Group phase II study (06893). Leukemia 11 Suppl 1: S24-7, 1997.

55. DeSimone J, Koshy M, Dorn L, Lavelle D, Bressler L, Molokie R, Talischy N: Maintenance of elevated fetal hemoglobin levels by decitabine during dose interval treatment of sickle cell anemia. Blood 99(11): 3905-8, 2002.

56. Koshy M, Dorn L, Bressler L, Molokie R, Lavelle D, Talischy N, Hoffman R, van Overveld W, DeSimone J: 2-deoxy 5-azacytidine and fetal hemoglobin induction in sickle cell anemia. Blood 96(7): 2379-84, 2000.

57. Saunthararajah Y, Hillery CA, Lavelle D, Molokie R, Dorn L, Bressler L, Gavazova S, Chen YH, Hoffman R, DeSimone J: Effects of 5-aza-2'-deoxycytidine on fetal hemoglobin levels, red cell adhesion, and hematopoietic differentiation in patients with sickle cell disease. Blood 102(12): 3865-70, 2003.

58. Momparler RL, Rivard GE, Gyger M: Clinical trial on 5-aza-2'-deoxycytidine in patients with acute leukemia. Pharmacol. Ther. 30(3): 277-86, 1985.

59. Momparler RL: Molecular, cellular and animal pharmacology of 5-aza-2'-deoxycytidine. Pharmacol. Ther. 30(3): 287-99, 1985.

60. Griffin JD, Lowenberg B: Clonogenic cells in acute myeloblastic leukemia. Blood 68(6): 1185-95, 1986.

61. Preisler HD, Azarnia N, Marinello M: Relationship of the growth of leukemic cells in vitro to the outcome of therapy for acute nonlymphocytic leukemia. Cancer Res. 44(4): 1712-17, 1984.

62. Freshney RI. Cloning hematopoietic cells in agar. In Culture of Animal Cells: A Manual of Basic Technology , 140-44, 1987.

63. Karsai A, Muller S, Platz S, Hauser MT: Evaluation of a homemade SYBR green I reaction mixture for real-time PCR quantification of gene expression. Biotechniques 32(4): 790-6, 2002.

64. Gottwald E, Muller O, Polten A: Semiquantitative reverse transcription-polymerase chain reaction with the Agilent 2100 Bioanalyzer. Electrophoresis 22(18): 4016-22, 2001.

65. Primeau M, Gagnon J, Momparler RL: Synergistic antineoplastic action of DNA methylation inhibitor 5-AZA-2'-deoxycytidine and histone deacetylase inhibitor depsipeptide on human breast carcinoma cells. Int. J. Cancer 103(2): 177-84, 2003.

66. Liu W, Saint DA: A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Anal. Biochem. 302(1): 52-9, 2002.

67. Kansas GS, Muirhead MJ, Dailey MO: Expression of the CD11/CD18, leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans. Blood 76(12): 2483-92, 1990.

68. Cameron EE, Baylin SB, Herman JG: p15(INK4B) CpG island methylation in primary acute leukemia is heterogeneous and suggests density as a critical factor for transcriptional silencing. Blood 94(7): 2445-51, 1999.

69. Aggerholm A, Guldberg P, Hokland M, Hokland P: Extensive intra- and interindividual heterogeneity of p15INK4B methylation in acute myeloid leukemia. Cancer Res. 59: 436-41, 1999.

70. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. U.S.A. 93(18): 9821-26, 1996.

71. Ho DH: Distribution of kinase and deaminase of 1-beta-D-arabinofuranosylcytosine in tissues of man and mouse. Cancer Res. 33(11): 2816-20, 1973.

72. Momparler RL, Samson J, Momparler LF, Rivard GE: Cell cycle effects and cellular pharmacology of 5-aza-2'-deoxycytidine. Cancer Chemother. Pharmacol. 13(3): 191-4, 1984.

73. Raza A, Preisler HD, Day R, Yasin Z, White M, Lykins J, Kukla C, Barcos M, Bennett J, Browman G: Direct relationship between remission duration in acute myeloid leukemia and cell cycle kinetics: a leukemia intergroup study. Blood 76(11): 2191-97, 1990.

74. Raza A, Preisler H, Lampkin B, Yousuf N, Tucker C, Peters N, White M, Kukla C, Gartside P, Siegrist C: Biological significance of cell cycle kinetics in 128 standard risk newly diagnosed patients with acute myelocytic leukemia. Br. J. Haematol. 79(1): 33-39, 1991.

75. Lubbert M: DNA methylation inhibitors in the treatment of leukemias, myelodysplastic syndromes and hemoglobinopathies: clinical results and possible mechanisms of action. Curr. Top. Microbiol. Immunol. 249: 135-64, 2000.

76. Ramani P, Shipley J: Recent advances in the diagnosis, prognosis and classification of childhood solid tumours. Br. Med. Bull. 52(4): 724-41, 1996.

77. Maddox AM, Johnston DA, Barlogie B, Haq M, Keating MJ, Freireich EJ: In vitro suppression of DNA synthesis by a remission induction agent and its correlation with response in adult acute leukemia. Eur. J. Cancer Clin. Oncol. 20 (4): 507-14, 1984.

78. Voso MT, D'Alo' F, Putzulu R, Mele L, Scardocci A, Chiusolo P, Latagliata R, Lo-Coco F, Rutella S, Pagano L, Hohaus S, Leone G: Negative prognostic value of glutathione S-transferase (GSTM1 and GSTT1) deletions in adult acute myeloid leukemia. Blood 100(8): 2703-7, 2002.

79. Houghton PJ, Stewart CF, Thompson J, Santana VM, Furman WL, Friedman HS: Extending principles learned in model systems to clinical trials design. Oncology (Huntingt.) 12(8) Suppl 6: 84-96, 1998.

80. Bovenzi V, Momparler RL: Antineoplastic action of 5-aza-2'-deoxycytidine and histone deacetylase inhibitor and their effect on the expression of retinoic acid receptor beta and estrogen receptor alpha genes in breast carcinoma cells. Cancer Chemother. Pharmacol. 48(1): 71-76, 2001.

81. Shaker S, Bernstein M, Momparler LF, Momparler RL: Preclinical evaluation of antineoplastic activity of inhibitors of DNA methylation (5-aza-2'-deoxycytidine) and histone deacetylation (trichostatin A, depsipeptide) in combination against myeloid leukemic cells. Leuk. Res. 27(5): 437-44, 2003.

82. Laliberte J, Marquez VE, Momparler RL: Potent inhibitors for the deamination of cytosine arabinoside and 5-aza-2'-deoxycytidine by human cytidine deaminase. Cancer Chemother. Pharmacol. 30(1): 7-11, 1992.

83. Pohlmann P, DiLeone LP, Cancella AI, Caldas AP, Dal Lago L, Campos OJ, Monego E, Rivoire W, Schwartsmann G: Phase II trial of cisplatin plus decitabine, a new DNA hypomethylating agent, in patients with advanced squamous cell carcinoma of the cervix. Am. J. Clin. Oncol. 25(5): 496-501, 2002.

84. Abbruzzese JL, Frost P: Studies on the mechanism of the synergistic interaction between 2'-deoxy-5-azacytidine and cisplatin. Cancer Chemother.Pharmacol. 30(1): 31-36, 1992.

85. Anzai H, Frost P, Abbruzzese JL: Synergistic cytotoxicity with 2'-deoxy-5-azacytidine and topotecan in vitro and in vivo. Cancer Res. 52(8): 2180-85, 1992.

86. Pinto A, Zagonel V: 5-Aza-2'-deoxycytidine (Decitabine) and 5-azacytidine in the treatment of acute myeloid leukemias and myelodysplastic syndromes: past, present and future trends. Leukemia 7 Suppl 1: 51-60, 1993.

87. Bradstock KF: The use of hematopoietic growth factors in the treatment of acute leukemia. Curr. Pharm. Des. 8(5): 343-55, 2002.

88. Karpf AR, Jones DA: Reactivating the expression of methylation silenced genes in human cancer. Oncogene 21(35): 5496-503, 2002.

89. Watt PM, Kumar R, Kees UR: Promoter demethylation accompanies reactivation of the HOX11 proto-oncogene in leukemia. Genes Chromosomes Cancer 29(4): 371-7, 2000.

90. Oberman M, Frader J: Dying children and medical research: access to clinical trials as benefit and burden. Am. J. Law Med. 29(2-3): 301-17, 2003.

91. Shah S, Weitman S, Langevin AM, Bernstein M, Furman W, Pratt C: Phase I therapy trials in children with cancer. J. Pediatr. Hematol. Oncol. 20(5): 431-38, 1998.

92. Kodish E, Stocking C, Ratain MJ, Kohrman A, Siegler M: Ethical issues in phase I oncology research: a comparison of investigators and institutional review board chairpersons. J. Clin. Oncol. 10(11):1810-16, 1992.

93. Daugherty C, Ratain MJ, Grochowski E, Stocking C, Kodish E, Mick R, Siegler M: Perceptions of cancer patients and their physicians involved in phase I trials. J. Clin. Oncol. 13(5):1062-72, 1995.

94. Hilden JM, Emanuel EJ, Fairclough DL, Link MP, Foley KM, Clarridge BC, Schnipper LE, Mayer RJ: Attitudes and practices among pediatric oncologists regarding end-of-life care: results of the 1998 American Society of Clinical Oncology survey. J. Clin. Oncol. 19(1): 205-12, 2001.

95. Estlin EJ, Cotterill S, Pratt CB, Pearson AD, Bernstein M: Phase I trials in pediatric oncology: perceptions of pediatricians from the United Kingdom Children's Cancer Study Group and the Pediatric Oncology Group. J. Clin. Oncol. 18(9): 1900-5, 2000.

ANNEXES

Authorization of the Editor

Authorization of the Co-authors

Presentation of this Project

Poster at the *44th annual meeting of the American Society of Hematology,* Philadelphia December 2002: *Expression of p15, p73 and E-Cadherin in Leukemic Cells after Treatment with Different Concentrations of 5-AZA-2'-Deoxycytidine.* NJ Farinha, S Shaker, M Lemaire, L Momparler, M Bernstein, RL Momparler. Blood, 100 (11): 322a-323a, 2002.